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(54) Lysosomal enzyme-cleavable antitumor drug conjugates.

(57) The present invention relates to drug-ligand conjugates wherein the drug is linked to the ligand through a protein peptide linker and a connector, a process for the preparation of said conjugates, method of controlling the growth of undesirable cells, pharmaceutical compositions, and intermediates thereof.

EP 0 624 377 A2

BACKGROUND OF THE INVENTION**1. Field of the Invention**

5 The present invention provides drug-ligand conjugates wherein the ligand is connected to the drug moiety through a peptide linker made up of a protein peptide specifier, a carboxylic acyl unit, and a self-immolating spacer, and which conjugates are activated by lysosomal enzymes.

2. Description of the Art

10 Bipartite compounds consisting of a carrier or linker moiety and a drug moiety are known. These compounds have been particularly useful in the formation of immunoconjugates directed against tumor associated antigens. In certain cases, however, bipartite compounds may be unstable due to the inherent nature of the bond linking the antibodies to the drug or due to the electronic or steric features of the drug moiety which may hinder hydrolysis of the bond by the desired target enzyme. Katzenellenbogen, J. Amer. Chem. Soc., (1981) 24: 479-480.

SUMMARY OF THE INVENTION

20 The present invention provides tumor specific, drug-ligand conjugates composed of a ligand, a drug, and a peptide linker, which conjugate is selectively activatable at the site of the tumor.

The drug-ligand conjugates of this invention comprise at least one drug molecule, a ligand capable of targeting a selected cell population, and a peptide linker which contains a carboxylic acyl, and a protein peptide specifier. The peptide linker may also contain a self-immolating spacer which spaces the protein peptide sequence and the drug.

The ligand is linked to the carboxylic acyl unit via a thioether-containing linker unit arm, which thioether bond is created by reaction of a sulfhydryl group on the ligand. In a preferred embodiment, the targeting ligand is attached directly to the peptide linker through a covalent thioether bond.

An aspect of the present invention provides drug conjugates which are selectively activatable at the site of the tumor.

Another aspect of the invention provides tumor-specific drug conjugates which are highly selective substrates for drug-activating enzymatic cleavage by one or more tumor-associated enzymes.

A further aspect of the invention provides tumor-specific drug conjugates wherein the activating enzyme is one which is present in the tumor in sufficient amounts to generate cytotoxic levels of free drug in the vicinity of the tumor.

Another aspect of the invention provides tumor-specific drug conjugates which tumor specificity arises solely from the ligand.

Another aspect of the invention provides tumor-specific drug conjugates which are stable to adventitious proteases in blood.

40 A still further aspect of the present invention provides a tumor-specific drug conjugate in accordance with the preceding aspects, which is considerably less toxic than the activated drug.

In another aspect the present invention provides a method for the production of the drug conjugates and pharmaceutical compositions and methods for delivering the conjugates to target cells in which a modification in biological process is desired, such as in the treatment of diseases such as cancer.

45 The present invention also provides a method for delivering to the site of tumor cells in a warm-blooded animal an active antitumor drug by administering to said warm-blooded animal the drug-ligand conjugate according to this invention.

In one embodiment the drug moiety is an anthracycline antibiotic, the ligand is an antibody, A is p-aminobenzyl-carbamoyl, Y is Phe, Z is Lys, and n is 5.

50 In a preferred embodiment the anthracycline drug moiety is doxorubicin, the ligand moiety is a chimeric antibody, A is p-aminobenzyl-carbamoyl, Y is Phe, Z is Lys, and n is 5.

In another preferred embodiment the drug moiety is taxol, the ligand is an antibody, Y is Phe, Z is Lys and n is 5.

In another preferred embodiment the drug moiety is mitomycin C, the ligand is an antibody, Y is Phe, Z is Lys and n is 5.

The above and other aspects of the present invention are achieved by derivatizing an antitumor agent linked to a ligand through a peptide linker, made up of a protein peptide sequence and a self-immolating spacer, at a reactive site appropriate for inhibiting the pharmacological activity of the antitumor agent to

thereby convert the antitumor agent into a pharmacologically inactive peptidyl derivative conjugate. The peptide linker has an amino acid residue sequence specifically tailored so as to render the peptidyl derivative a selective substrate for drug-activating enzymatic cleavage by one or more lysosomal proteases, such as cathepsin B, C or D. The enzymatic cleavage reaction will remove the peptide linker moiety from the drug conjugate and effect release of the antitumor agent in pharmacologically active form selectively at the tumor site. In comparison with ligand-drug linkers which rely on simple acid hydrolysis for drug release this new method provides significantly less systemic toxicity due to premature linker hydrolysis in the blood, consequently a greater amount of drug is delivered to the tumor site, and the method results in a longer storage life and simplified handling conditions for the conjugate.

The drug-ligand conjugates of the present invention show significantly less systemic toxicity than biparte conjugates and free drug. The conjugates of the invention retain both specificity and therapeutic drug activity for the treatment of a selected target cell population. They may be used in a pharmaceutical composition, such as one comprising a pharmaceutically effective amount of a compound of Formula (I) below, associated with a pharmaceutically acceptable carrier, diluent or excipient.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the expression of the BR96 in the L2987 lung line, the A2780 ovarian line and the HCT116 colon line.

20 Figure 2 shows the potency of the BR96-doxorubicin conjugate and unconjugated doxorubicin.

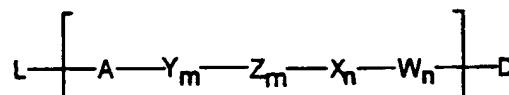
DETAILED DESCRIPTION OF THE INVENTION

The following detailed description is provided so that the invention may be more fully understood.

25 The present invention provides novel drug-ligand conjugates composed of a ligand capable of targeting a selected cell population, and a drug connected to the ligand by a peptide linker. The peptide linker is made up of a carboxylic acyl unit and a protein peptide sequence. The peptide linker may also contain a self-immolating spacer, which spaces the drug and the protein peptide sequence.

30 The ligand molecule can be an immunoreactive protein such as an antibody, or fragment thereof, a non-immunoreactive protein, or peptide ligand such as bombesin or, a binding ligand recognizing a c II associated receptor such as a lectin, or any protein or peptide that possesses a reactive sulphydryl group (-SH) or can be modified to contain such a sulphydryl group. The carboxylic acyl unit is linked to the ligand via a thioether bond, and the drug is linked to the linker via a functional group selected from primary or secondary amine, hydroxyl, sulphydryl, carboxyl, aldehyde or ketone.

35 A conjugate of the present invention is represented by general Formula (I):



40 Formula (I)

45 in which

- D is a drug moiety;
- L is a ligand;
- A is a carboxylic acyl unit
- Y is an amino acid;
- Z is an amino acid;
- X is a self-immolative spacer;
- W is a self-immolative spacer;
- m is an integer of 1, 2, 3, 4, 5 or 6.
- n is an integer of 0 or 1.

50 55 For a better understanding of the invention, the drugs, ligands, peptides and spacers will be discussed individually. The synthesis of the conjugates will be explained.

It will be understood that in the following detailed description and appended claims, the abbreviations and nomenclature employed are those which are standard in amino acid and peptide chemistry, and that all

the amino acids referred to are in the L-form unless otherwise specified.

The abbreviations used in the present application, unless otherwise indicated are as follows:

AcOH: acetic acid; Ala: L-alanine; Alloc: allyloxycarbonyl; Arg: L-arginine; Boc: t-butyloxycarbonyl; Cit: L-citrulline; DBU: diazobicycloundecene; DCC: dicyclohexylcarbodiimide; DCI: direct chemical ionization; DCU: dicyclohexylurea; DIEA: diisopropylethylamine; DMAP: 4-dimethylaminopyridine; DME: 1,2-dimethoxyethane; DOX: doxorubicin; DTT: dithiothreitol; EEDQ: N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; EtOAc: ethyl acetate; FAB: fast atom bombardment; Fmoc: fluorenylmethoxycarbonyl; GABA: γ -aminobutyric acid; Gly: glycine; HOEt: N-hydroxybenzotriazole; HRMS: high resolution mass spectroscopy; LDL: low density lipoprotein; Ile: L-isoleucine; Leu: L-leucine; Lys: L-lysine; MC: 6-maleimidocaproyl; MMA: mitomycin A; MMC: mitomycin C; Mtr: 4-methoxytrityl; NHS: N-hydroxysuccinimide; NMP: N-methylpyrrolidinone; PABC: p-aminobenzyl-carbamoyl; PAB-OH: p-aminobenzyl alcohol; Phe:L-phenylalanine; PNP: p-nitrophenol; TFA: trifluoroacetic acid; THF: tetrahydrofuran; Trp:L-tryptophan; Val: L-valine; Z: benzyloxycarbonyl.

15 THE PEPTIDE LINKER

The peptide linker of the present invention is made up of a carboxylic acyl unit, and a protein peptide sequence. The linker may also contain a self-immolating spacer which spaces the drug and the protein peptide sequence.

In the conjugate of Formula I, the peptide linker is represented by "A--Y--Z--X--W" in which "A" is the carboxylic acyl unit, "Y" and "Z" are each amino acids and together form the protein peptide sequence, and "X" and "W" are individually self-immolating spacers which spaces the protein peptide and the drug.

THE PROTEIN PEPTIDE SEQUENCE In the conjugate of Formula I,

Y is at least one amino acid selected from the group consisting of alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan and proline, preferably phenylalanine or valine; and

Z is at least one amino acid selected from the group consisting of lysine, lysine protected with acetyl or formyl, arginine, arginine protected with tosyl or nitro groups, histidine, ornithine, ornithine protected with acetyl or formyl, and citrulline, preferably lysine, or citrulline.

The amino acid residue sequence is specifically tailored so that it will be selectively enzymatically cleaved from the resulting peptidyl derivative drug-conjugate by one or more of the tumor-associated proteases.

The amino acid residue chain length of the peptide linker preferably ranges from that of a dipeptide to that of a tetrapeptide. It will be understood, however, that peptide linkers as long as eight amino acid residues may also suitably be employed.

The following group of exemplary peptide linker groups, are named in order to illustrate further the conjugates of the present invention:

Phe-Lys, Val-Lys, Phe-Phe-Lys, D-Phe-Phe-Lys, Gly-Phe-Lys, Ala-Lys, Val-Cit, Phe-Cit, Leu-Cit, Ile-Cit, Trp-Cit, Phe-Ala, Gly-Phe-Leu-Gly, Ala-Leu-Ala-Leu, Phe-N⁹-tosyl-Arg, and Phe-N⁹-Nitro-Arg.

Specific examples of the preferred embodiment of peptide sequences include Phe-Lys, Val-Lys, Val-Cit, and D-Phe-L-Phe-Lys.

Numerous specific peptide linker molecules suitable for use in the present invention can be designed and optimized in their selectivity for enzymatic cleavage by a particular tumor-associated protease. The preferred peptide linkers for use in the present invention are those which are optimized toward the proteases, cathepsin B, C and D. Cathepsin B was shown to release DOX from the conjugate at pH 5.3 (37°C) with (*t_{1/2}* = 3.0 hrs.).

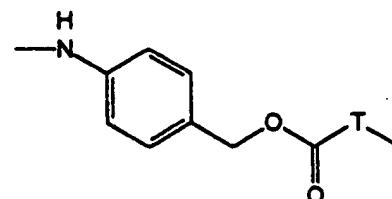
THE SPACER The drug-conjugates in accordance with the present invention may employ an intermediate self-immolative spacer moiety which spaces and covalently links together the drug moiety and the protein peptide moiety. A self-immolative spacer may be defined as a bifunctional chemical moiety which is capable of covalently linking together two spaced chemical moieties into a normally stable tripartite molecule, releasing one of said spaced chemical moieties from the tripartite molecule by means of enzymatic cleavage; and following said enzymatic cleavage, spontaneously cleaving from the remainder of the molecule to release the other of said spaced chemical moieties. In accordance with the present invention, the self-immolative spacer is covalently linked at one of its ends to the protein peptide moiety and covalently linked at its other end to the chemical active site of the drug moiety whose derivatization inhibits pharmacological activity, so as to space and covalently link together the protein peptide moiety and the drug moiety into a tripartite molecule which is stable and pharmacologically inactive in the absence of the target enzyme, but which is enzymatically cleavable by such target enzyme at the bond covalently linking the spacer moiety and the protein peptide moiety thereby effect release of the protein peptide.

moiety from the tripartite molecule. Such enzymatic cleavage, in turn, will activate the self-immolating character of the spacer moiety and initiate spontaneous cleavage of the bond covalently linking the spacer moiety to the drug moiety, to thereby effect release of the drug in pharmacologically active form.

In the conjugate of Formula I,

5 X is a spacer moiety which spaces and covalently links together the drug moiety and the amino acid, in which the spacer is linked to the drug moiety via the T moiety, and which may be represented by the compounds of Formulae (III), (IV), (V) or (VI):

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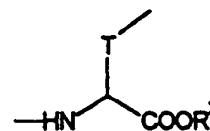
Formula (III)

20 in which T is O, N or S,

-HN-R¹-COT- Formula (IV)

25 in which T is O, N or S, and
R¹ is C₁-C₅ alkylene

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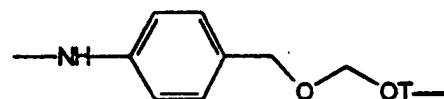


Formula (V)

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(J. Med. Chem., 27: 1447 (1984))
in which T is O, N or S, and
R² is H or C₁-C₅ alkyl,

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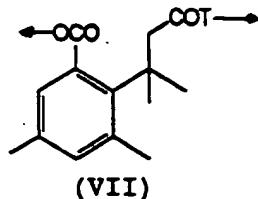
Formula (VI)

50 or

W is a spacer moiety represented by the Formula (VII)

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10 wherein T is O, S or N, and wherein the arrows indicate the linkage to the neighbouring groups.

As used herein "C₁-C₅ alkyl" is meant to include branched or unbranched hydrocarbon chain having, unless otherwise noted, one to five carbon atoms, including but not limited to methyl, ethyl, isopropyl, n-propyl, sec-butyl, isobutyl, n-butyl, n-pentyl, i-pentyl, neopentyl and the like. As used herein "C₁-C₅ alkylene" is meant to include brached or unbranched hydrocarbon chain having, unless otherwise noted, one to five carbon atoms, including but not limited to methylene, ethylene, isopropylene, n-propylene, sec-butylene, isobutylene, n-butylene, n-pentylene, i-pentylene, neopentylene, 1,1-dimethyl-propylene, and the like.

15 A preferred spacer moiety suitable for use in the present invention is derived from PABC and represented by the Formula (IIIa):

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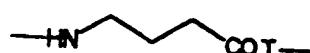


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Formula (IIIa)

Another preferred spacer moiety suitable for use in the present invention is derived from GABA and represented by the Formula (IVa):

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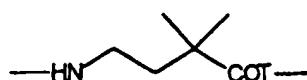


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Formula (IVa)

Yet another preferred spacer moiety suitable for use in the present invention is derived from α,α -dimethyl GABA and represented by the Formula (IVb):

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Formula (IVb)

Another preferred spacer moiety suitable for use in the present invention is derived from β,β -dimethyl GABA and represented by the Formula (IVc):

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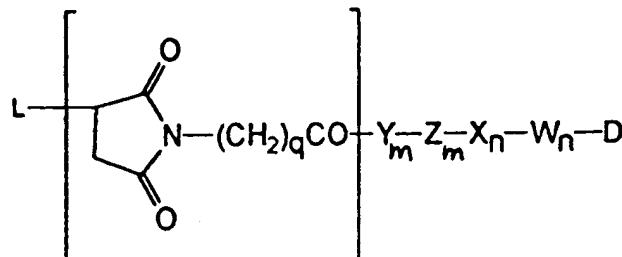


wherein T in each of the above-mentioned formulae originates from the drug moiety.

THE CARBOXYLIC ACYL UNIT In the conjugate of Formula (I), the carboxylic acyl unit "A" is linked to the ligand via a sulfur atom derived from the ligand. Representative of conjugates of this invention are compounds of Formulae (IXa), (IXb), (IXc), (IXd) and (IXe), which "A" is the compound in brackets.

15

20



A

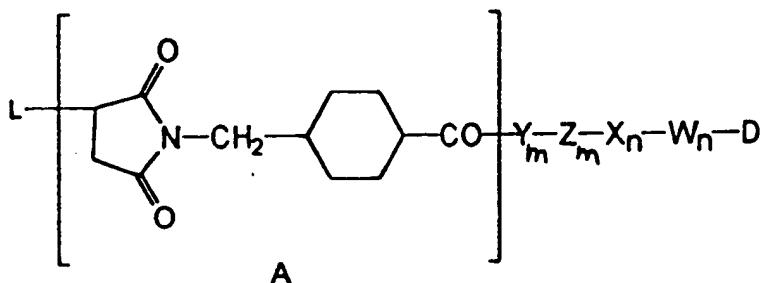
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Formula (IXa)

in which q is 1-10, and L, Y, Z, X, W, D, n and m are as previously defined;

30

35



A

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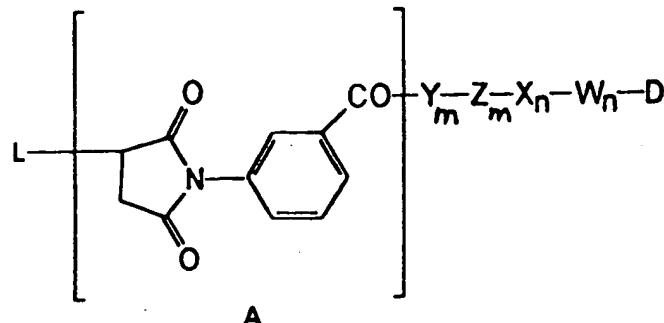
Formula (IXb)

made from succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (Pierce Catalog p. E-15 (1992)), wherein L, Y, Z, X, W, D, n and m are as previously defined;

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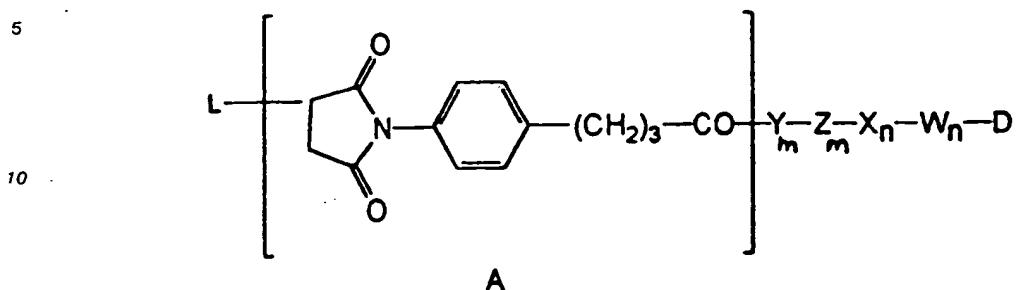
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A

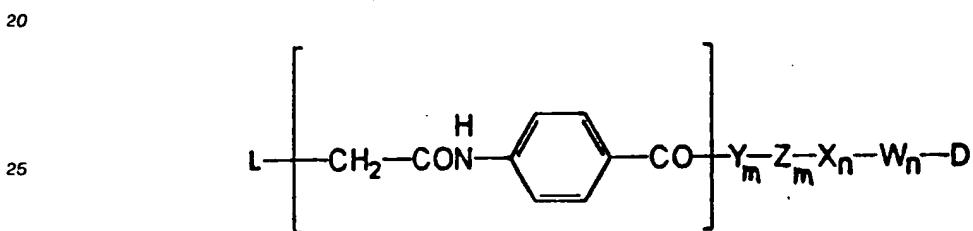
Formula (IXc)

made from m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (Pierce Catalog p. E-16 (1992)), wherein L, Y, Z, X, W, D, n and m are as previously defined;



15 Formula (IXd)

made from succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) (Pierce catalog p. E-18 (1992), wherein L, Y, Z, X, W, D, n and m are as previously defined;

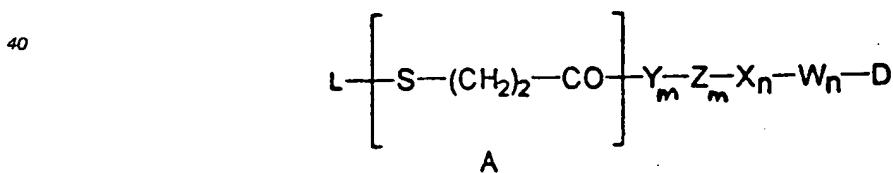


A

30 Formula (IXe)

made from N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB) (Pierce catalog p. E-17 (1992)), wherein L, Y, Z, X, W, D, n and m are as previously defined; or

35 A is a compound that joins up to the peptide and is linked to the ligand via a sulfur atom derived from the ligand, and a sulfur atom derived from the carboxylic acyl unit to form a dithio link. Representative of conjugates of this invention are compounds of Formulae (Xa), (Xb) and (Xc)

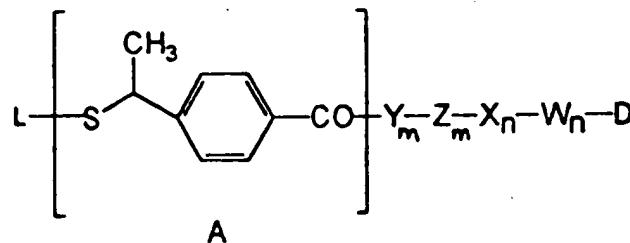


Formula (Xa)

made from N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pierce catalog p. E-13 (1992)), wherein L, Y, Z, X, W, D, n and m are as previously defined;

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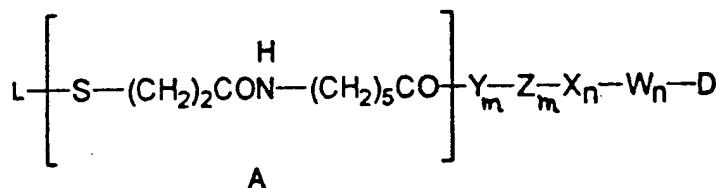
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10 Formula (Xb)

made from 4-succinimidylloxycarbonyl- α -methyl- α -(2-pyridylidithio)-toluene (SMPT) (Pierce catalog p. E-12
(1992)), wherein L, Y, Z, X, W, D, n and m are as previously defined; and

15



20

25 Formula (Xc)

25

made from long chain SPDP (Pierce catalog p. E-14 (1992), wherein L, Y, Z, X, W, D, n and m are as previously defined.

30 THE DRUG

The drug conjugates of the present invention are effective for the usual purposes for which the corresponding drugs are effective, and have superior efficacy because of the ability, inherent in the ligand, to transport the drug to the desired cell where it is of particular benefit. Further, because the conjugates of the invention can be used for modifying a given biological response, the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a protein such as tumor necrosis factor.

The preferred drugs for use in the present invention are cytotoxic drugs, particularly those which are used for cancer therapy. Such drugs include, in general, DNA damaging agents, anti-metabolites, natural products and their analogs. Preferred classes of cytotoxic agents include, for example, the enzyme inhibitors such as dihydrofolate reductase inhibitors, and thymidylate synthase inhibitors, DNA intercalators, DNA cleavers, topoisomerase inhibitors, the anthracycline family of drugs, the vinca drugs, the mitomycins, the bleomycins, the cytotoxic nucleosides, the pteridine family of drugs, diarylenes, the podophyllotoxins, differentiation inducers, and taxols. Particularly useful members of those classes include, for example, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosidine, actinomycin, daunorubicin, doxorubicin, mitomycin C, mitomycin A, carminomycin, aminopterin, tallysomycin, podophyllotoxin and podophyllotoxin derivatives such as etoposide or etoposide phosphate, vinblastine, vincristine, vindesine, taxol, taxotere retinoic acid, butyric acid, N⁸-acetyl spermidine, camptothecin, and their analogues.

As noted previously, one skilled in the art may make chemical modifications to the desired compound in order to make reactions of that compound more convenient for purposes of preparing conjugates of the invention.

In the conjugate of Formula I,

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group by means of which the drug backbone is bonded to the protein peptide linker, said functional group selected from the group consisting of a primary or secondary amine, hydroxyl, sulfhydryl, carboxyl, aldehyde or a ketone.

Representative of said amino containing drugs are mitomycin-C, mitomycin-A, daunorubicin, doxorubicin, aminopterin, actinomycin, bleomycin, 9-amino camptothecin, N⁸-acetyl spermidine, 1-(chloroethyl)-1,2-dimethanesulfonyl hydrazide, tallsomycin, cytarabine and derivatives thereof.

Representative of said alcohol group containing drugs are etoposide, camptothecin, taxol, esperamicin, 5 1,8-dihydroxy-bicyclo[7.3.1] trideca-4-9-diene-2,6-diyne-13-one, (U.S. Patent 5,198,560), podophyllotoxin, anguidine, vincristine, vinblastine, morpholine-doxorubicin, n-(5,5-diacetoxy-pentyl) doxorubicin, and derivatives thereof.

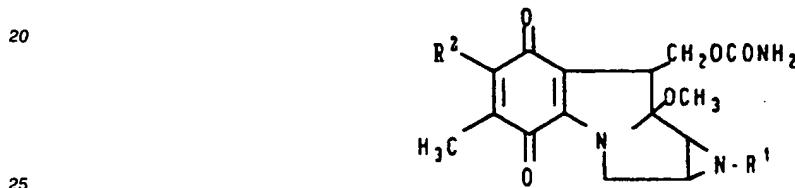
Representative of said sulphydryl containing drugs are esperamicin and 6-mercaptopurine, and derivatives thereof.

10 Representative of said carboxyl containing drugs are methotrexate, camptothecin (ring-opened form of the lactone), butyric acid, retinoic acid, and derivatives thereof.

Representative of said aldehyde and ketone containing drugs are anguidine and anthracyclines such as doxorubicin, and derivatives thereof.

15 A highly preferred group of cytotoxic agents for use as drugs in the present invention include drugs of the following formulae:

THE MITOMYCIN GROUP OF FORMULA (1):



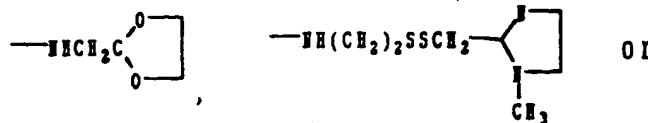
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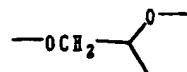
in which

R¹ is hydrogen or methyl;
 R² is -NH₂, -OCH₃, -O(CH₂)₂OH, -NH(CH₂)₂SS(CH₂)₂NHAc,
 35 -NHCH-C≡CH, -NH(CH₂)₂SS(C₆H₄)NO₂,
 -O(CH₂)₂SS(CH₂)₂OH, -N=CH-NHOCH₃,
 -NH(C₆H₄)OH,
 -NH(CH₂)₂SS(CH₂)₂NHCO(CH₂)₂CH(NH₂)COOH

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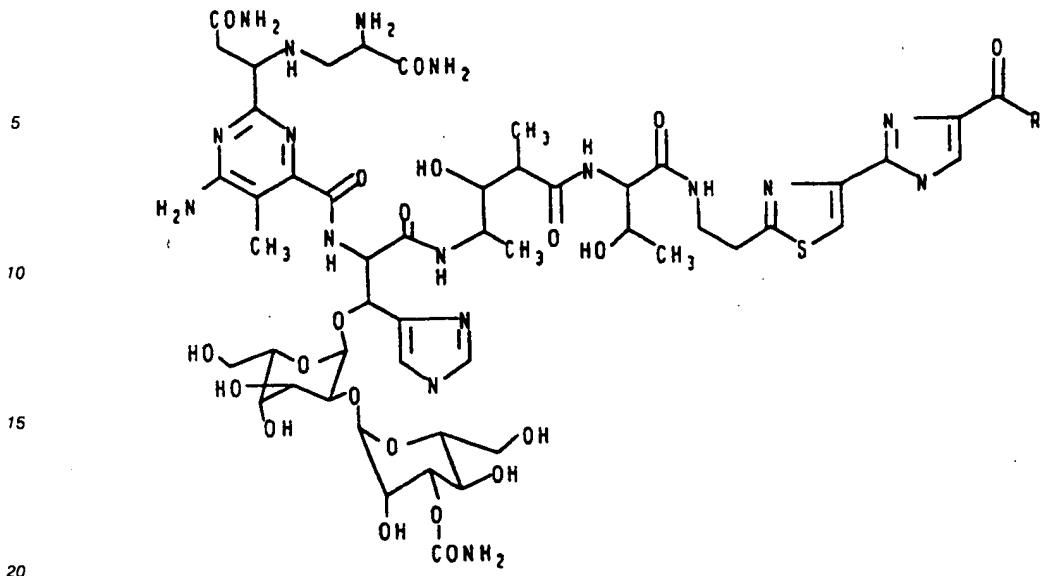
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THE BLEOMYCIN GROUP OF FORMULA (2):

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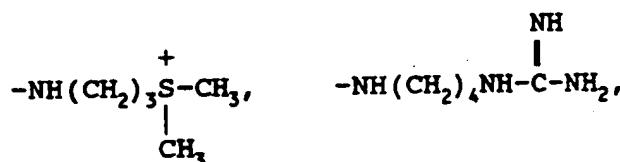


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in which

R¹ is hydroxy, amino, C₁-C₃ alkylamino,
di(C₁-C₃ alkyl)amino, C₄-C₆ polymethylene amino,

30



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or

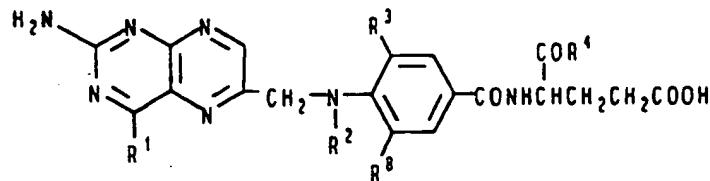
$$-\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$$

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THE METHOTREXATE GROUP OF FORMULA (3):

5



10

in which

15 R¹ is amino or hydroxy;
 R² is hydrogen or methyl;
 R³ is hydrogen, fluoro, chloro, bromo or iodo;
 R⁴ is hydroxy or a moiety which completes a salt of the carboxylic acid.

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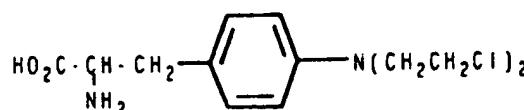
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MELPHALAN OF FORMULA (4):

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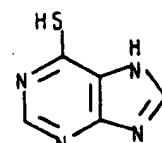


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6-MERCAPTOPURINE OF FORMULA (5):

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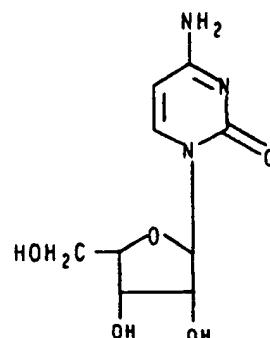
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(5)

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A CYTOSINE ARABINOSIDE OF FORMULA (6):

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(6)

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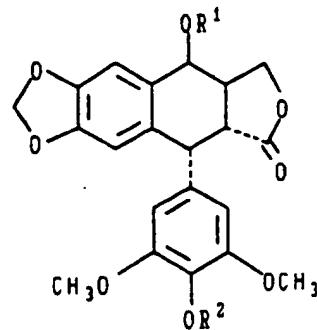
THE PODOPHYLLOTOXINS OF FORMULA (7):

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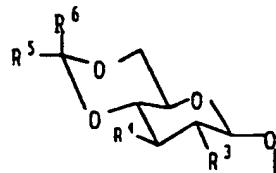
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(7)

wherein

20 R² is hydrogen,
R¹ is hydrogen or

25



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wherein

R³ is NH₂, OH, OCH₃, NH(C₁-C₃ alkyl) or N(C₁-C₃ alkyl)₂
 R⁴ is OH, or NH₂,
 R⁵ is methyl or thienyl,
 35 R⁶ is hydrogen or methyl,
 or a phosphate salt thereof.

As used herein "C₁-C₃ alkyl" means a straight or branched carbon chain having from one to three carbon atoms; examples include methyl, ethyl, n-propyl and isopropyl.

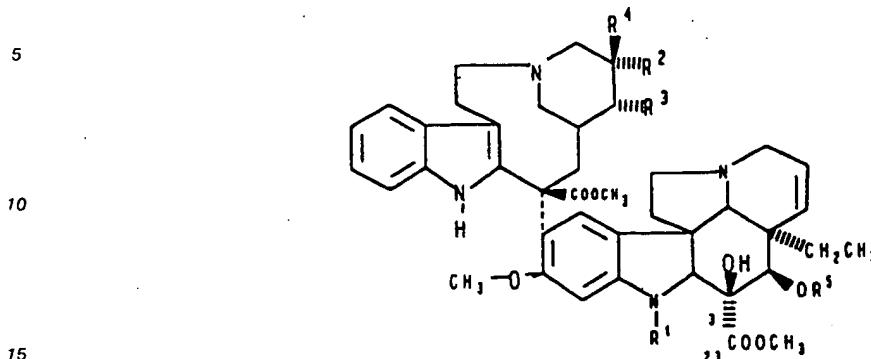
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THE VINCA ALKALOID GROUP OF DRUGS OF FORMULA (8):



(8)

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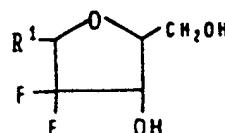
in which

 R^1 is H, CH_3 or CHO ;when R^2 and R^3 are taken singly, R^3 is H, and one of R^4 and R^2 is ethyl and the other is H or OH;when R^2 and R^3 are taken together with the carbons to which they are attached, they form an oxirane25 ring in which case R^4 is ethyl; R^5 is hydrogen, (C_1 - C_3 alkyl)-CO, or chlorosubstituted (C_1 - C_3 alkyl)-CO.As used herein " C_1 - C_3 alkyl" means a straight or branched carbon chain having from one to three carbon atoms; examples include methyl, ethyl, n-propyl and isopropyl.

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DIFLUORONUCLEOSIDES OF FORMULA (9):

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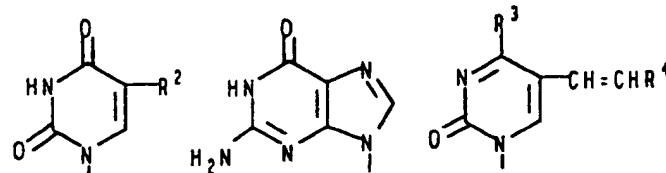


(9)

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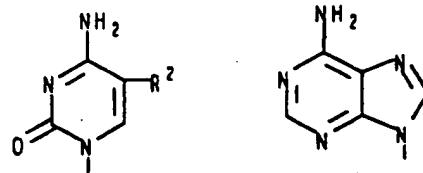
in which R^1 is a base of one of the formulae:

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in which

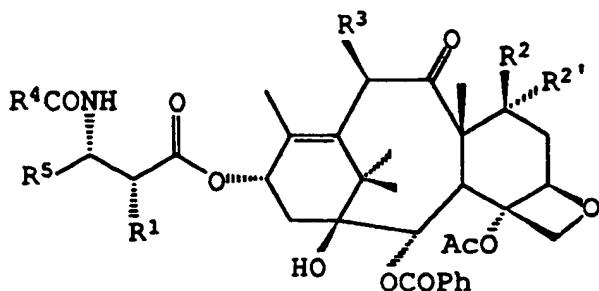
- R² is hydrogen, methyl, bromo, fluoro, chloro, or iodo;
- R³ is -OH or -NH₂;
- R⁴ is hydrogen, bromo, chloro, or iodo.

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TAXOLS OF FORMULA (10):

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(10)

wherein

- 25 R¹ is hydroxy;
- R² is hydrogen or hydroxy;
- R^{2'} is hydrogen or fluoro;
- R³ is hydrogen, hydroxy, or acetoxy;
- R⁴ is aryl, substituted aryl, C₁-6 alkyl, C₂-6 alkenyl, C₂-6 alkynyl or t-butoxy;
- 30 R⁵ is C₁-6 alkyl, C₂-6 alkenyl, C₂-6 alkynyl, or -Z-R⁶;
- Z is a direct bond, C₁-6 alkyl or C₂-6 alkenyl;
- R⁶ is aryl, substituted aryl, C₃-6 cycloalkyl, thienyl or furyl.

As used herein, "alkyl" means a straight or branched saturated carbon chain having from one to six carbon atoms; examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, t-butyl, n-pentyl, sec-pentyl, isopentyl, and n-hexyl. "Alkenyl" means a straight or branched carbon chain having at least one carbon-carbon double bond, and having from two to six carbon atoms; examples include ethenyl, propenyl, isopropenyl, butenyl, isobutenyl, pentenyl, and hexenyl. "Alkynyl" means a straight or branched carbon chain having at least one carbon-carbon triple bond, and from two to six carbon atoms; examples include ethynyl, propynyl, butynyl, and hexynyl. "Aryl" means aromatic hydrocarbon having from six to ten carbon atoms; examples include phenyl and naphthyl. "Substituted aryl" means aryl substituted with at least one group selected from C₁-6 alkanoyloxy, hydroxy, halogen, C₁-6 alkyl, trifluoromethyl, C₁-6 alkoxy, aryl, C₂-6 alkenyl, C₁-6 alkanoyl, nitro, amino, and amido.

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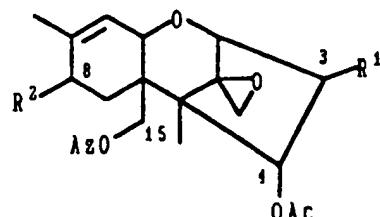
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ANGUIDINES OF FORMULA (11):

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(11)

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wherein

R¹ is OH or OR² is H or O

Anguidine can be targeted at the C-3, C-4, C-8 or C-15 positions, as an ester or hydrazone.

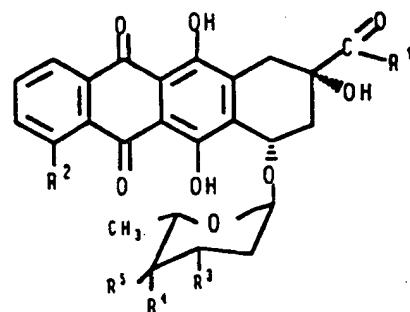
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THE ANTHRACYCLINES ANTIBIOTICS OF FORMULA (12):

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(12)

wherein

40 R¹ is -CH₃, -CH₂OH, -CH₂OCO(CH₂)₃CH₃ or -CH₂OCOCH(OC₂H₅)₂R² is -OCH₃, -OH or -HR³ is -NH₂, -NHCOCF₃, 4-morpholinyl, 3-cyano-4-morpholinyl, 1-piperidinyl, 4-methoxy-1-piperidinyl, benzylamine, dibenzylamine, cyanomethylamine, 1-cyano-2-methoxyethyl amine, or NH-(CH₂)₄-CH(OAc)₂;45 R⁴ is -OH, -OTHP, or -H; andR⁵ is -OH or -H provided that R⁵ is not -OH when R⁴ is -OH or -OTHP.

One skilled in the art understands that structural Formula (12) includes compounds which are drugs, or are derivatives of drugs, which have acquired in the art different generic or trivial names. Table I, which follows, represents a number of anthracycline drugs and their generic or trivial names and which are especially preferred for use in the present invention.

50 Of the compounds shown in Table I, the most highly preferred drug is Doxorubicin. Doxorubicin (also referred to herein as "DOX") is that anthracycline of Formula (1) in which R₁ is -CH₂OH, R₃ is -OCH₃, R₄ is -NH₂, R₅ -OH, and R₆ is -H.

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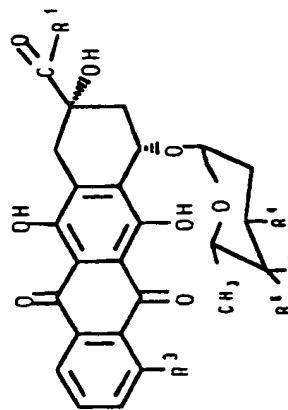


Table I

Compound	R ¹	R ³	R ⁴	R ⁵	R ⁶
Daunorubicin*	CH ₃	OCH ₃	NH ₂	OH	H
Doxorubicin	CH ₂ OH	OCH ₃	NH ₂	OH	H
Detorubicin	CH ₂ OOCCH(OCH ₂ H ₅) ₂	OCH ₃	NH ₂	OH	H
Carminomycin	CH ₃	OH	NH ₂	OH	H
Idarubicin	CH ₃	H	NH ₂	H	OH
Epirubicin	CH ₂ OH	OCH ₃	NH ₂	H	H
Esorubicin	CH ₂ OH	OCH ₃	NH ₂	OTHP	H
THP	CH ₂ OOC(CH ₂) ₃ CH ₃	OCH ₃	NH ₂	OH	H
AD-32			NHCOCF ₃		
Morpholino-Dox	CH ₂ OH	OCH ₃	—N—Cyclohexyl	OH	H
Cyano-morpholino-Dox	CH ₂ OH	OCH ₃	—N—Cyclohexyl	OH	H
DAPDox	CH ₂ OH	OCH ₃	—NH(CH ₂) ₂ CH(OAc) ₂	OH	H

*"Daunomycin" is an alternate name for daunorubicin

The most highly preferred drugs are the taxol, mitomycin C, and anthracycline antibiotic agents of Formula (12), described previously.

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THE LIGAND

The "ligand" includes within its scope any molecule that specifically binds or reactively associates or complexes with a receptor or other receptive moiety associated with a given target cell population. This cell reactive molecule, to which the drug reagent is linked via the linker in the conjugate, can be any molecule that binds to, complexes with or reacts with the cell population sought to be therapeutically or otherwise biologically modified and, which possesses a free reactive sulfhydryl (-SH) group or can be modified to contain such a sulfhydryl group. The cell reactive molecule acts to deliver the therapeutically active drug moiety to the particular target cell population with which the ligand reacts. Such molecules include, but are not limited to, large molecular weight proteins such as, for example, antibodies, smaller molecular weight proteins, polypeptide or peptide ligands, and non-peptidyl ligands.

The non-immunoreactive protein, polypeptide, or peptide ligands which can be used to form the conjugates of this invention may include, but are not limited to, transferrin, epidermal growth factors ("EGF"), bombesin, gastrin, gastrin-releasing peptide, platelet-derived growth factor, IL-2, IL-6, tumor growth factors ("TGF"), such as TGF- α and TGF- β , vaccinia growth factor ("VGF"), insulin and insulin-like growth factors I and II. Non-peptidyl ligands may include, for example, carbohydrates, lectins, and apoprotein from low density lipoprotein.

The immunoreactive ligands comprise an antigen-recognizing immunoglobulin (also referred to as "antibody"), or an antigen-recognizing fragment thereof. Particularly preferred immunoglobulins are those immunoglobulins which can recognize a tumor-associated antigen. As used, "immunoglobulin" may refer to any recognized class or subclass of immunoglobulins such as IgG, IgA, IgM, IgD, or IgE. Preferred are those immunoglobulins which fall within the IgG class of immunoglobulins. The immunoglobulin can be derived from any species. Preferably, however, the immunoglobulin is of human, murine, or rabbit origin. Further, the immunoglobulin may be polyclonal or monoclonal, preferably monoclonal.

As noted, one skilled in the art will appreciate that the invention also encompasses the use of antigen recognizing immunoglobulin fragments. Such immunoglobulin fragments may include, for example, the Fab', F(ab')₂, Fv or Fab fragments, or other antigen recognizing immunoglobulin fragments. Such immunoglobulin fragments can be prepared, for example, by proteolytic enzyme digestion, for example, by pepsin or papain digestion, reductive alkylation, or recombinant techniques. The materials and methods for preparing such immunoglobulin fragments are well-known to those skilled in the art. See generally, Parham, *J. Immunology*, 131, 2895 (1983); Lamoyi et al., *J. Immunological Methods*, 56, 235 (1983); Parham, id., 53, 133 (1982); and Matthew et al., id., 50, 239 (1982).

The immunoglobulin can be a "chimeric antibody" as that term is recognized in the art. Also, the immunoglobulin may be a "bifunctional" or "hybrid" antibody, that is, an antibody which may have one arm having a specificity for one antigenic site, such as a tumor associated antigen while the other arm recognizes a different target, for example, a hapten which is, or to which is bound, an agent lethal to the antigen-bearing tumor cell. Alternatively, the bifunctional antibody may be one in which each arm has specificity for a different epitope of a tumor associated antigen of the cell to be therapeutically or biologically modified. In any case, the hybrid antibodies have a dual specificity, preferably with one or more binding sites specific for the hapten of choice or one or more binding sites specific for a target antigen, for example, an antigen associated with a tumor, an infectious organism, or other disease state.

Biological bifunctional antibodies are described, for example, in European Patent Publication, EPA 0 105 360, to which those skilled in the art are referred. Such hybrid or bifunctional antibodies may be derived, as noted, either biologically, by cell fusion techniques, or chemically, especially with cross-linking agents or disulfide bridge-forming reagents, and may be comprised of whole antibodies and/or fragments thereof. Methods for obtaining such hybrid antibodies are disclosed, for example, in PCT application WO83/03679, published October 27, 1983, and published European Application EPA 0 217 577, published April 8, 1987, both of which are incorporated herein by reference. Particularly preferred bifunctional antibodies are those biologically prepared from a "polydoma" or "quadroma" or which are synthetically prepared with cross-linking agents such as bis-(maleimido)-methyl ether ("BMME"), or with other cross-linking agents familiar to those skilled in the art.

In addition the immunoglobulin may be a single chain antibody ("SCA"). These may consist of single chain Fv fragments ("scFv") in which the variable light ("V_L") and variable heavy ("V_H") domains are linked by a peptide bridge or by disulfide bonds. Also, the immunoglobulin may consist of single V_H domains (dAbs) which possess antigen-binding activity. See, e.g., G. Winter and C. Milstein, *Nature*, 349, 295 (1991); R. Glockshuber et al., *Biochemistry* 29, 1362 (1990); and, E. S. Ward et al., *Nature* 341, 544 (1989).

Especially preferred for use in the present invention are chimeric monoclonal antibodies, preferably those chimeric antibodies having specificity toward a tumor associated antigen. As used herein, the term

"chimERIC antibody" refers to a monoclonal antibody comprising a variable region, i.e. binding region, from one source or species and at least a portion of a constant region derived from a different source or species, usually prepared by recombinant DNA techniques. Chimeric antibodies comprising a murine variable region and a human constant region are especially preferred in certain applications of the invention, particularly 5 human therapy, because such antibodies are readily prepared and may be less immunogenic than purely murine monoclonal antibodies. Such murine/human chimeric antibodies are the product of expressed immunoglobulin genes comprising DNA segments encoding murine immunoglobulin variable regions and DNA segments encoding human immunoglobulin constant regions. Other forms of chimeric antibodies encompassed by the invention are those in which the class or subclass has been modified or changed from 10 that of the original antibody. Such "chimeric" antibodies are also referred to as "class-switched antibodies". Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques now well known in the art. See, e.g., Morrison, S.L. et al., Proc. Nat'l Acad. Sci., 81, 6851 (1984).

Encompassed by the term "chimeric antibody" is the concept of "humanized antibody", that is those 15 antibodies in which the framework or "complementarity determining regions ("CDR") have been modified to comprise the CDR of an immunoglobulin of different specificity as compared to that of the parent immunoglobulin. In a preferred embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody". See, e.g., L. Riechmann et al., Nature 332, 323 (1988); M. S. Neuberger et al., Nature 314, 268 (1985). Particularly preferred CDR'S correspond to those representing 20 sequences recognizing the antigens noted above for the chimeric and bifunctional antibodies. The reader is referred to the teaching of EPA 0 239 400 (published September 30, 1987), incorporated herein by reference, for its teaching of CDR modified antibodies.

One skilled in the art will recognize that a bifunctional-chimeric antibody can be prepared which would have the benefits of lower immunogenicity of the chimeric or humanized antibody, as well as the flexibility, especially for therapeutic treatment, of the bifunctional antibodies described above. Such bifunctional-chimeric antibodies can be synthesized, for instance, by chemical synthesis using cross-linking agents 25 and/or recombinant methods of the type described above. In any event, the present invention should not be construed as limited in scope by any particular method of production of an antibody whether bifunctional, chimeric, bifunctional-chimeric, humanized, or an antigen-recognizing fragment or derivative thereof.

In addition, the invention encompasses within its scope immunoglobulins (as defined above) or 30 immunoglobulin fragments to which are fused active proteins, for example, an enzyme of the type disclosed in Neuberger, et al., PCT application, WO86/01533, published March 13, 1986. The disclosure of such products is incorporated herein by reference.

As noted, "bifunctional", "fused", "chimeric" (including humanized), and "bifunctional-chimeric" (including 35 humanized) antibody constructions also include, within their individual contexts constructions comprising antigen recognizing fragments. As one skilled in the art will recognize, such fragments could be prepared by traditional enzymatic cleavage of intact bifunctional, chimeric, humanized, or chimeric-bifunctional antibodies. If, however, intact antibodies are not susceptible to such cleavage, because of the nature of the construction involved, the noted constructions can be prepared with immunoglobulin fragments used as the starting materials; or, if recombinant techniques are used, the DNA sequences, themselves, can be tailored 40 to encode the desired "fragment" which, when expressed, can be combined *in vivo* or *in vitro*, by chemical or biological means, to prepare the final desired intact immunoglobulin "fragment". It is in this context, therefore, that the term "fragment" is used.

Furthermore, as noted above, the immunoglobulin (antibody), or fragment thereof, used in the present 45 invention may be polyclonal or monoclonal in nature. Monoclonal antibodies are the preferred immunoglobulins, however. The preparation of such polyclonal or monoclonal antibodies now is well known to those skilled in the art who, of course, are fully capable of producing useful immunoglobulins which can be used in the invention. See, e.g., G. Kohler and C. Milstein, Nature 256, 495 (1975). In addition, hybridomas and/or monoclonal antibodies which are produced by such hybridomas and which are useful in the practice 50 of the present invention are publicly available from sources such as the American Type Culture Collection ("ATCC") 12301 Parklawn Drive, Rockville, Maryland 20852 or, commercially, for example, from Boehringer-Mannheim Biochemicals, P.O. Box 50816, Indianapolis, Indiana 46250.

Particularly preferred monoclonal antibodies for use in the present invention are those which recognize tumor associated antigens. Such monoclonal antibodies, are not to be so limited, however, and may include, for example, the following:

	<u>Antigen Site Recognized</u>	<u>Monoclonal Antibodies</u>	<u>Reference</u>
	Lung Tumors	KS1/4	N. M. Varki, <u>et al.</u> , <u>Cancer Res.</u> , 44:681, 1984
5		534, F8; 604A9	F. Cuttitta, <u>et al.</u> , in: G. L. Wright (ed) <u>Monoclonal Antibodies and Cancer</u> , Marcel Dekker, Inc., NY., p. 161, 1984.
10	Squamous Lung	G1, LuCa2, LuCa3, LuCa4	Kyoizumi <u>et al.</u> , <u>Cancer Res.</u> , 45:3274, 1985.
	Small Cell Lung Cancer	TFS-2	Okabe <u>et al.</u> , <u>Cancer Res.</u> , 45:1930, 1985.
15	Colon Cancer	11.285.14 14.95.55	G. Rowland, <u>et al.</u> , <u>Cancer Immunol. Immunother.</u> , 19:1, 1985
		NS-3a-22, NS-10 NS-19-9, NS-33a NS-52a, 17-1A	Z. Steplewski, <u>et al.</u> , <u>Cancer Res.</u> , 41:2723, 1981.
20	Carcinoembryonic	MoAb 35 or ZCE025	Acolla, R.S. <u>et al.</u> , <u>Proc. Natl. Acad. Sci., (USA)</u> , 77:563, 1980.
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	<u>Antigen Site Recognized</u>	<u>Monoclonal Antibodies</u>	<u>Reference</u>
5	Melanoma	9.2.27	T. F. Bumol and R. A. Reisfeld, <u>Proc. Natl. Acad. Sci. (USA)</u> , 79:1245, 1982.
	p97	96.5	K. E. Hellstrom, <u>et al.</u> , <u>Monoclonal Antibodies and Cancer</u> , <u>loc. cit.</u> p. 31.
10	Antigen T65	T101	Boehringer-Mannheim, P.O. Box 50816, Indianapolis, IN 46250
	Ferritin	Antiferrin	Boehringer-Mannheim, P.O. Box 50816, Indianapolis, IN 46250
15		R24	W. G. Dippold, <u>et al.</u> , <u>Proc. Natl. Acad. Sci. (USA)</u> , 77: 6114, 1980
20	Neuroblastoma	P1 153/3	R.H. Kennet and F. Gilbert, <u>Science</u> , 203:1120, 1979.
		MIN 1	J. T. Kemshead in <u>Monoclonal Antibodies and Cancer</u> , <u>loc. cit.</u>
25		UJ13A	p. 49. Goldman <u>et al.</u> , <u>Pediatrics</u> , 105:252, 1984.
	Glioma	BF7, GE2, CG12	N. de Tribolet, <u>et al.</u> , in <u>Monoclonal Antibodies and Cancer</u> , <u>loc. cit.</u> p. 81
30	Ganglioside	L6	I. Hellstrom <u>et al.</u> , <u>Proc. Natl. Acad. Sci. (U.S.A.)</u> 83:7059 (1986); U.S. Pat. Nos. 4,906,562, issued March 6, 1990 and 4,935,495, issued June 19, 1990.
35		Chimeric L6	U.S. Ser. No. 07/923,244, filed Oct. 27, 1986, equivalent to PCT Patent Publication, WO 88/03145, published May 5, 1988.
40	Lewis Y	BR64	U. S. Ser. Nos. 07/289,635, filed December 22, 1988, and U. S. Ser. No. 07/443,696, filed Nov. 29, 1989, equivalent to European Patent Publication, EP A 0 375 562, published June 27, 1990.
45	fucosylated Lewis Y	BR96, Chimeric BR96	U.S. Ser. Nos. 07/374,947, filed June 30, 1989, and U. S. Ser. No. 07/544,246, filed June 26, 1990, equivalent to PCT Patent Publication, WO 91/00295, published January 10, 1991.
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<u>Antigen Site Recognized</u>	<u>Monoclonal Antibodies</u>	<u>Reference</u>
Breast Cancer	B6.2, B72.3	D. Colcher, <u>et al.</u> , in <u>Monoclonal Antibodies and Cancer</u> , loc. cit. p. 121.
Osteogenic Sarcoma	791T/48, 791T/36	M. J. Embleton, <u>ibid</u> , p. 181
Leukemia	CALL 2 anti-idiotype	C. T. Teng, <u>et al.</u> , <u>Lancet</u> , 1:01, 1982 R. A. Miller, <u>et al.</u> , <u>N. Eng. J. Med.</u> , 306: 517, 1982
Ovarian Cancer	OC 125	R. C. Bast, <u>et al.</u> , <u>J. Clin. Invest.</u> , 68: 1331, 1981.
Prostrate Cancer	D83.21, P6.2, Turp-27	J. J. Starling, <u>et al.</u> , in <u>Monoclonal Antibodies and Cancer</u> , loc. cit., p.253
Renal Cancer	A6R, D5D	P. H. Lange, <u>et al.</u> , <u>Surgery</u> , 98:143, 1985.

In the most preferred embodiment, the ligand containing conjugate is derived from chimeric antibody BR96, "ChiBR96", disclosed in U.S. Ser. No. 07/544,246, filed June 26, 1990, and which is equivalent to PCT Published Application, WO 91/00295, published January 10, 1991. ChiBR96 is an internalizing murine/human chimeric antibody and is reactive, as noted, with the fucosylated Lewis Y antigen expressed by human carcinoma cells such as those derived from breast, lung, colon, and ovarian carcinomas. The hybridoma expressing chimeric BR96 and identified as ChiBR96 was deposited on May 23, 1990, under the terms of the Budapest Treaty, with the American Type Culture Collection ("ATCC"), 12301 Parklawn Drive, Rockville, Maryland 20852. Samples of this hybridoma are available under the accession number ATCC HB 10460. ChiBR96 is derived, in part, from its source parent, BR96. The hybridoma expressing BR96 was deposited, on February 21, 1989, at the ATCC, under the terms of the Budapest Treaty, and is available under the accession number HB 10036. The desired hybridoma is cultured and the resulting antibodies are isolated from the cell culture supernatant using standard techniques now well known in the art. See, e.g., "Monoclonal Hybridoma Antibodies : Techniques and Applications", Hurell (ed.) (CRC Press, 1982).

Thus, as used "immunoglobulin" or "antibody" encompasses within its meaning all of the immunoglobulin/antibody forms or constructions noted above.

Preparation of the Conjugates

The conjugates of the present invention may be constructed by attaching the drug moiety to the antibody through a linker made up of a peptide sequence which may be cleaved by the lysosomal proteases cathepsin B, C and D, and a self-immolating spacer.

A process for preparing the compound of the present invention is one wherein a solution of the antibody in a phosphate buffer or PBS was treated with a solution of dithiothreitol (DTT) at 25-45°C, for about 1-10 hours under N₂. The solution was then diafiltered against phosphate buffered saline (PBS) for 1/2 to 12 hours depending on the size of diafiltration cell and volume of solution under N₂, until the effluent is free of SH groups, then treated with the appropriate amount of peptide-PABC-drug [based on the number of SH groups in the Mab (determined by Ellman titration)] in distilled water, at 0 ±10°C for 15 minutes to 8 hours. The solution was then dialyzed against PBS for about 24 hours, at room temperature, then filtered and the filtrate was shaken for 15 minutes to 8 hours at room temperature with Biobeads, followed by another filtration.

Schemes 1-11 show the synthesis of model compounds that were tested with cathepsin B in order to determine the optimal characteristics of the linker including the peptide sequence, self-immolating spacer, and attachment to antibody.

Scheme 12 shows the synthesis of the linker compound MC-Ph-Lys-PABC-DOX (50) which is conjugated to the antibody carrier. The NHS active ester of Fmoc-Phe (43) was coupled to N'-Mtr-Lys (42) in an organic/aqueous solvent mixture to give the dipeptide Fmoc-Ph-N'-Mtr-Lys (44). This in turn was

coupled to p-aminobenzyl alcohol using EEDQ resulting in alcohol 45. The Fmoc group was removed with di thylamine, and the free N-terminal Phe was coupled to MC-NHS to give maleimidopeptide alcohol 47. Addition of bis-p-nitrophenyl carbonate provided the activated carbonate 48 and the p-nitrophenyl group was displaced by DOX in NMP at room temperature. The resulting substrate MC-Phe-N'-Mtr-Lys-PABC-DOX (49) was deprotected in quantitative yield by treatment with dichloroacetic acid/anisole in CH₂Cl₂ for 1 hour to give 50.

5 Scheme 13 shows the synthesis of a MMC-containing linker compound MC-Phe-Lys-PABC-MMC (52) from activated carbonate 48. The aziridine nitrogen of MMC is not nucleophilic enough to directly displace the p-nitrophenol of 48 but, in the presence of a 10-fold excess of HOBr, some of the corresponding HOBr active ester forms, and is active enough to react with MMC. Chloroacetic acid is used instead of dichloroacetic acid for the deprotection of 51 because of acid sensitivity of MMC.

10 Scheme 14 shows the preparation of a taxol containing linker compound MC-Phe-Lys-PABC-7-taxol (55). Maleimidopeptide alcohol 47 was treated with 2'-Mtr-taxol-7-chloroformate (prepared from 53) to give MC-phe-N'-Mtr-Lys-PABC-7-Taxol (54). This was deprotected with chloroacetic acid to give 55.

15 Scheme 15 shows the synthesis of a citrulline containing linker compound MC-Val-Cit-PABC-DOX (62) which is carried out essentially as described above for 49 and requires no side chain deprotection.

20 Scheme 16 shows the preparation of a linker compound containing an added aminocaproyl spacer designed so as to move the site of enzymatic cleavage away from the bulky antibody. MC-NH-C-Phe-Lys-PABC-DOX (72) was prepared using procedures essentially identical to those used in the synthesis of 50 and 55.

Scheme 17 shows the synthesis of a MMC-containing linker compound MC-Phe-Lys-GABA-MMC (78) which incorporates a GABA spacer in place of PABC. This was prepared essentially as described for 52 above.

25 Scheme 18 shows the synthesis of a potential protease-active prodrug of cortisone, Z-Phe-Lys-Cortisone (81). This was prepared essentially as described for MC-Phe-Lys-PABC-DOX (50).

Scheme 19 shows the synthesis of a linker compound containing taxol-2'-ethyl carbonate, an active prodrug of taxol.

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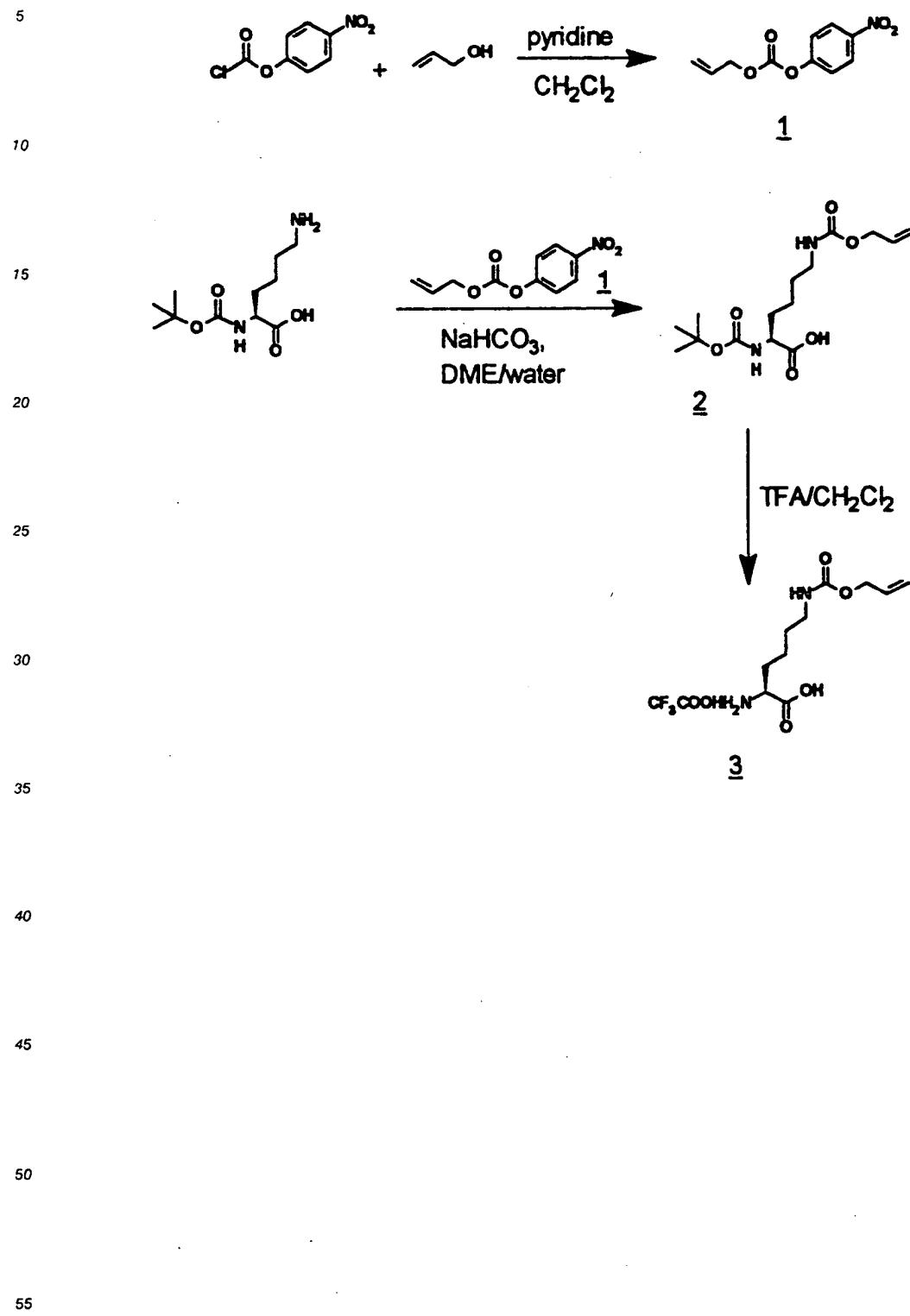
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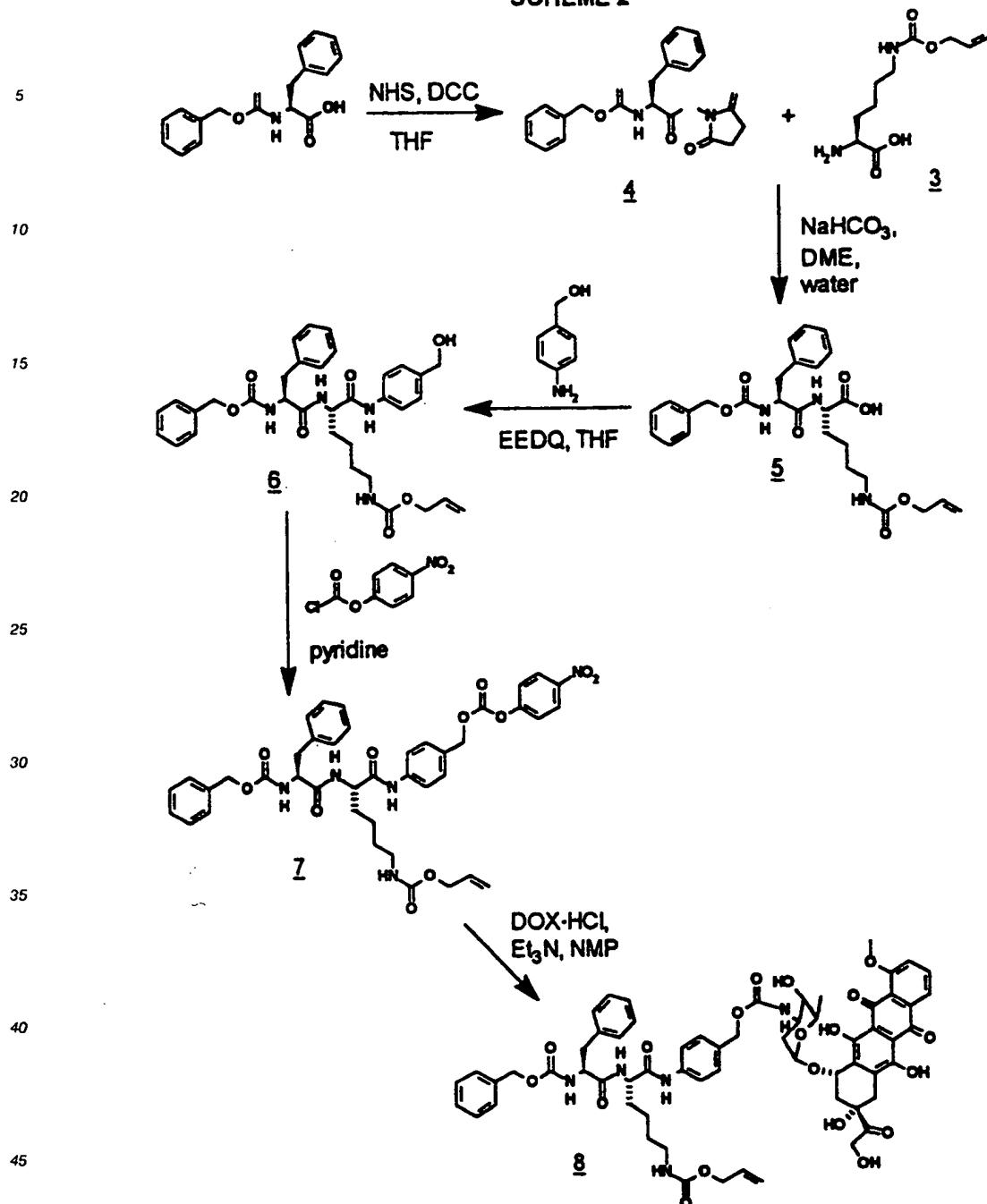
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SCHEME 1



SCHEME 2

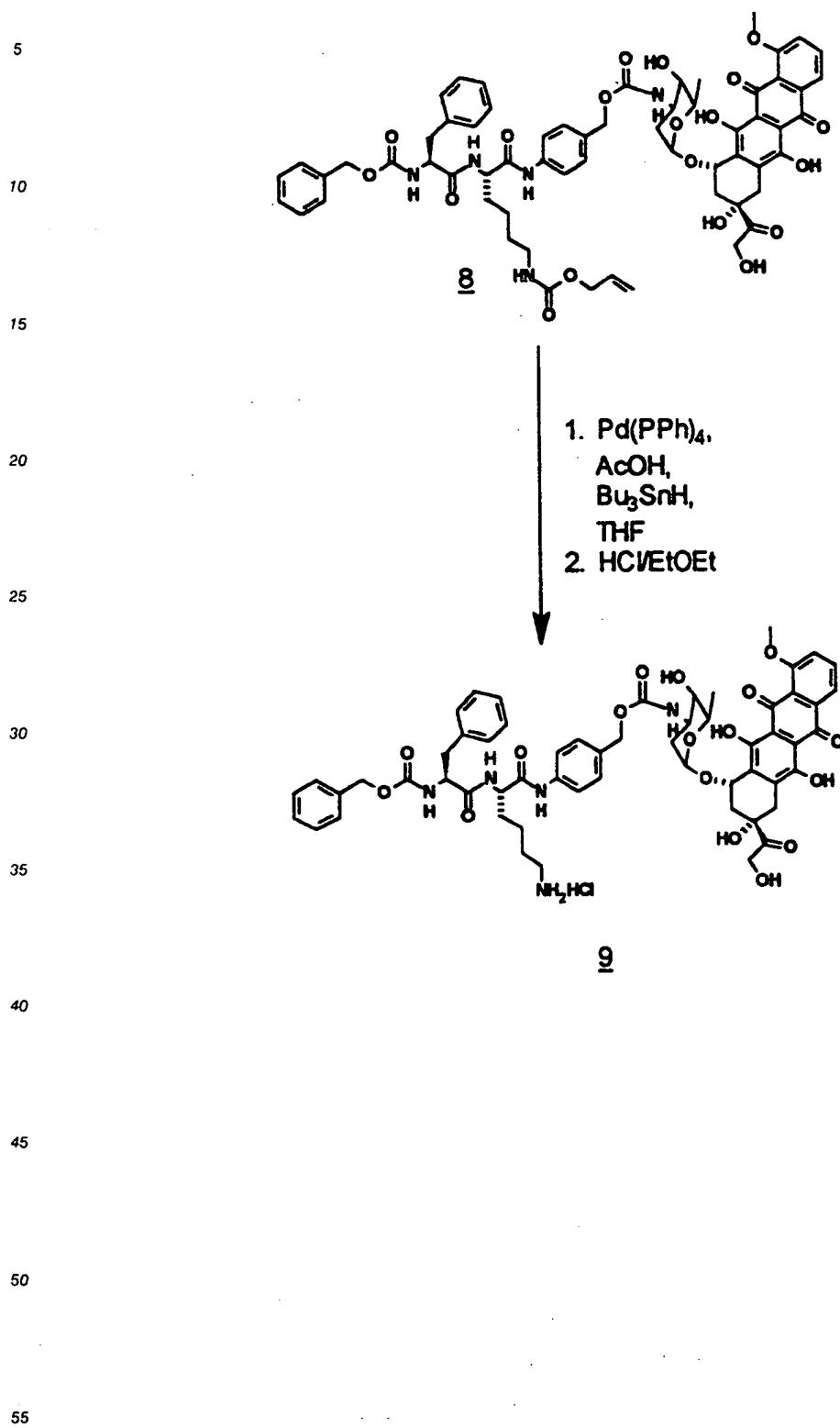


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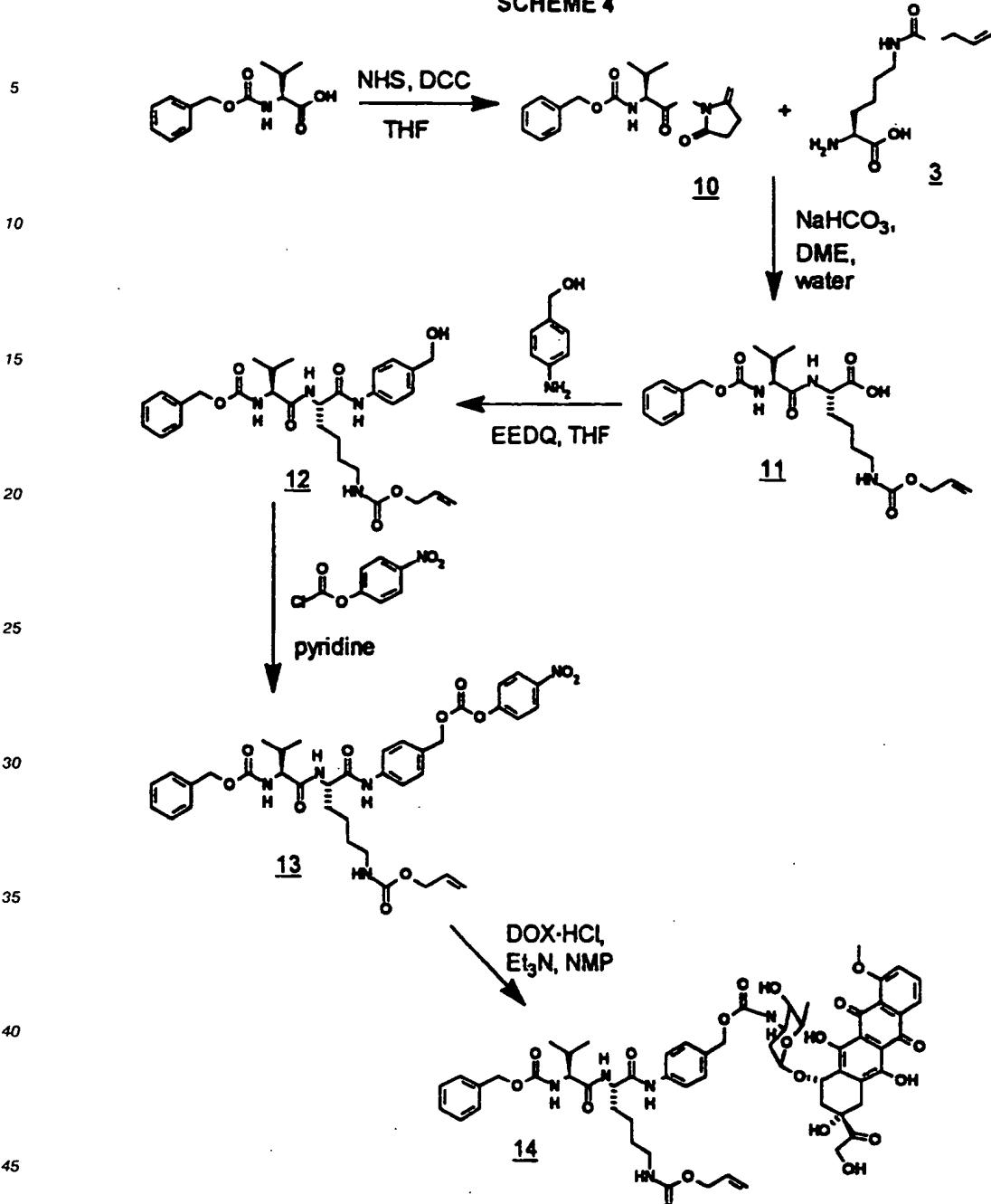
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SCHEME 3



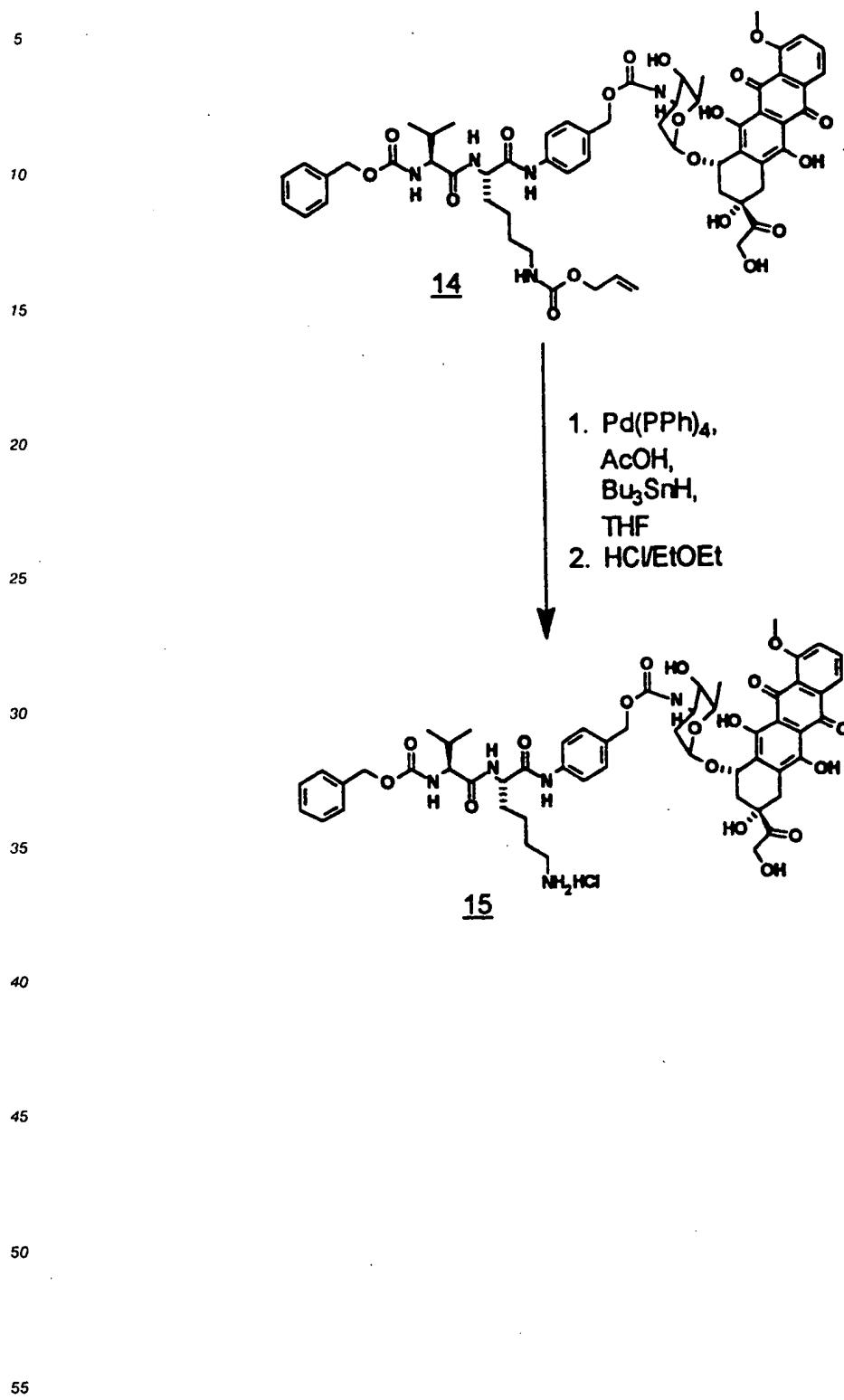
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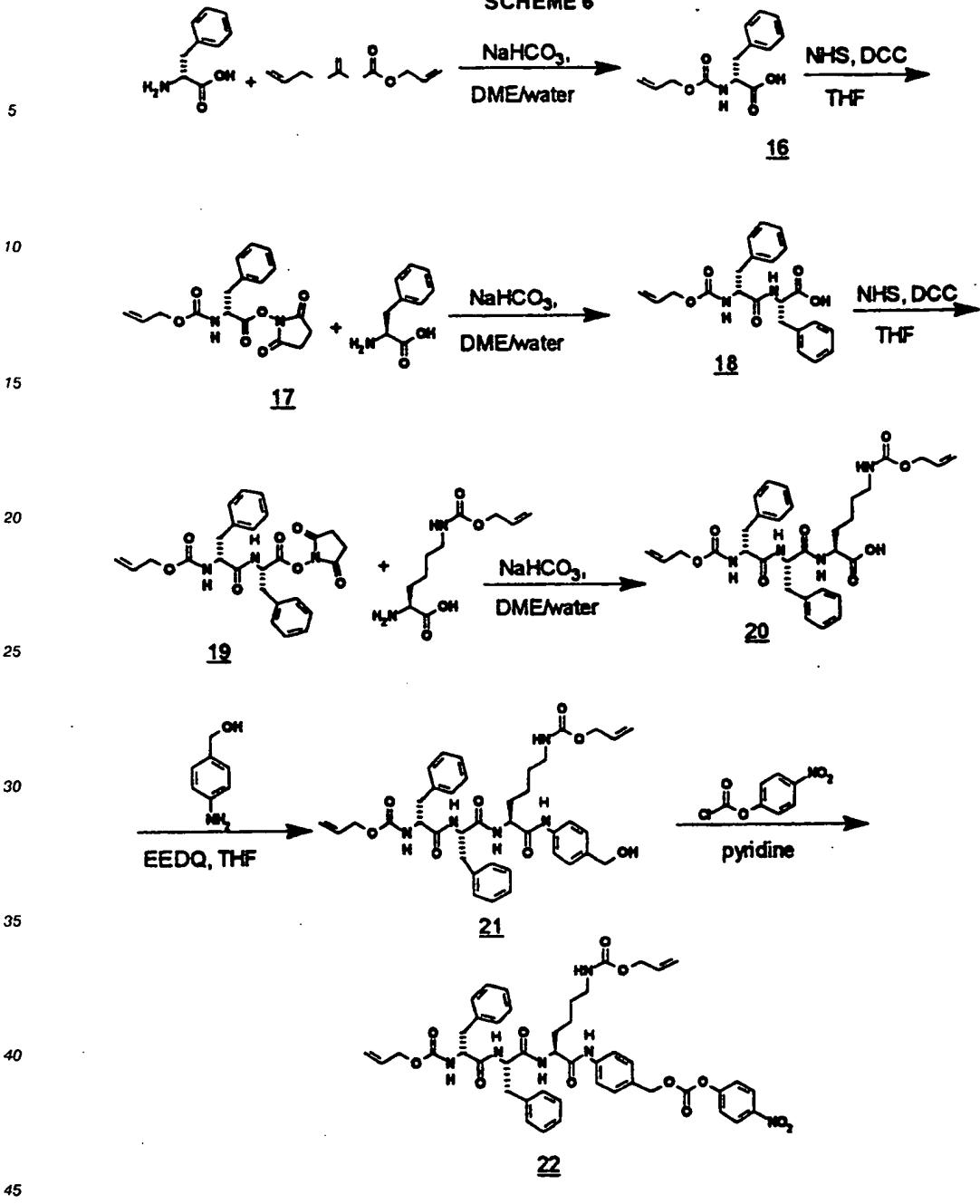
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SCHEME 5



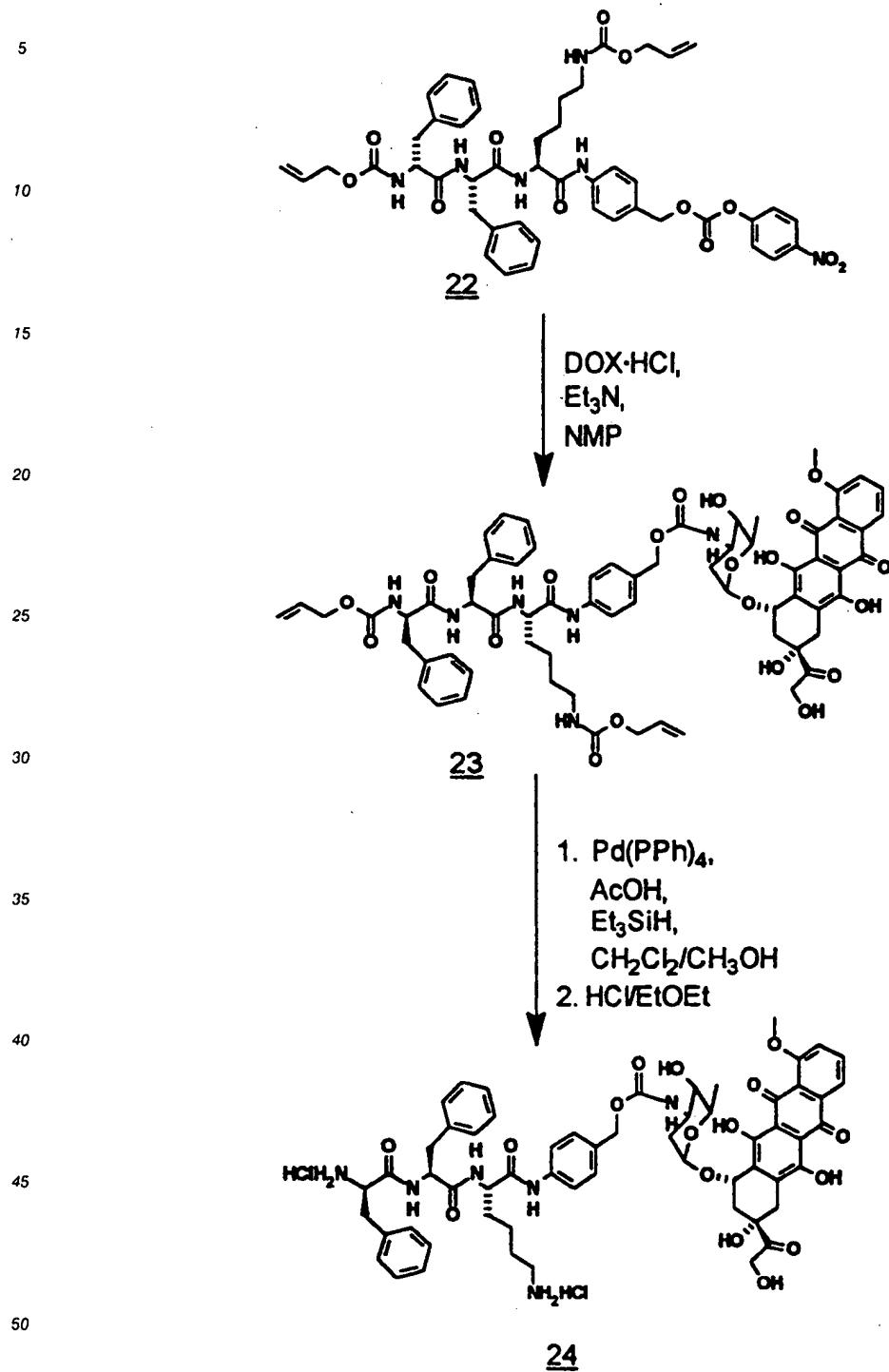
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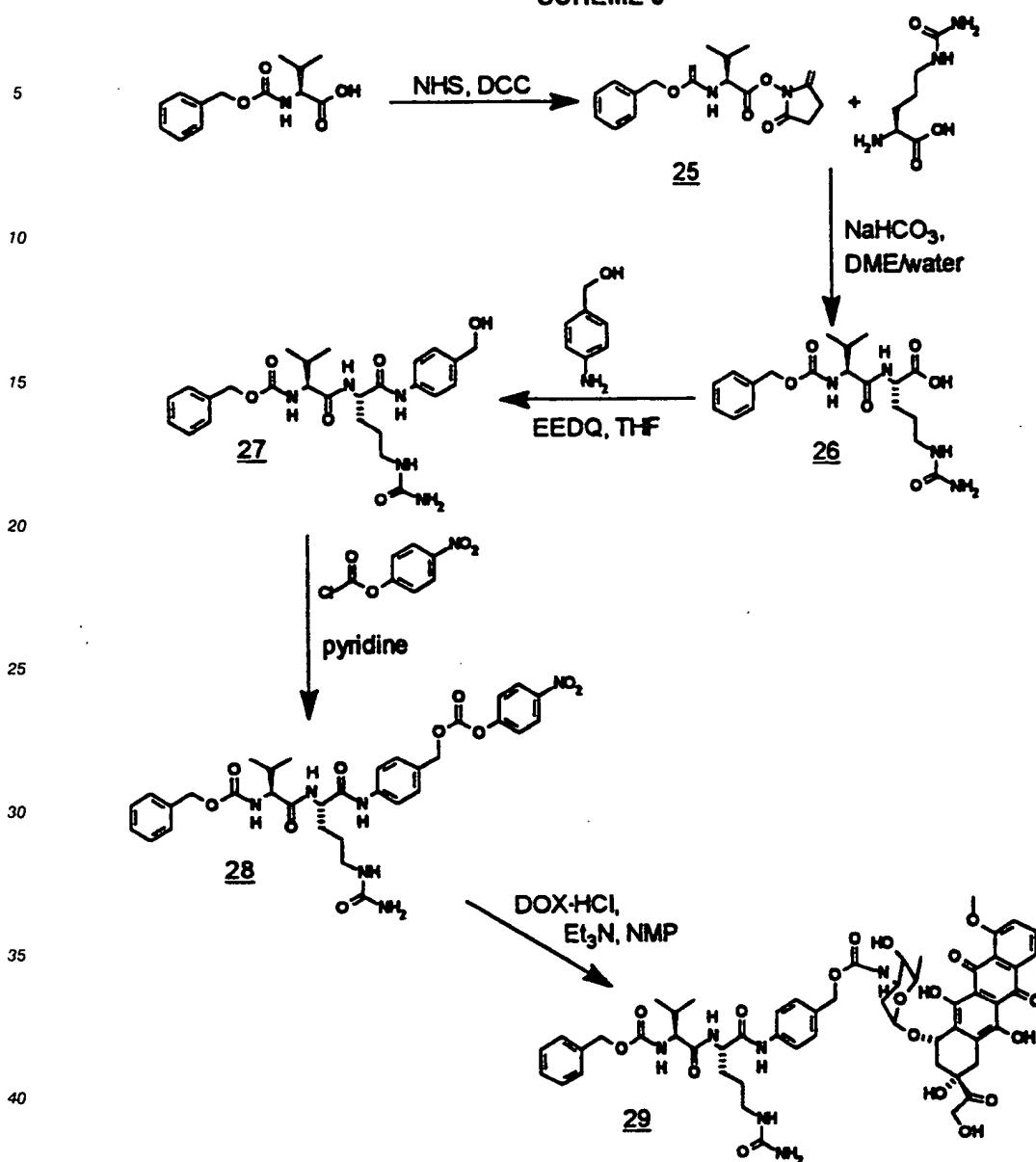
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SCHEME 7



SCHEME 8

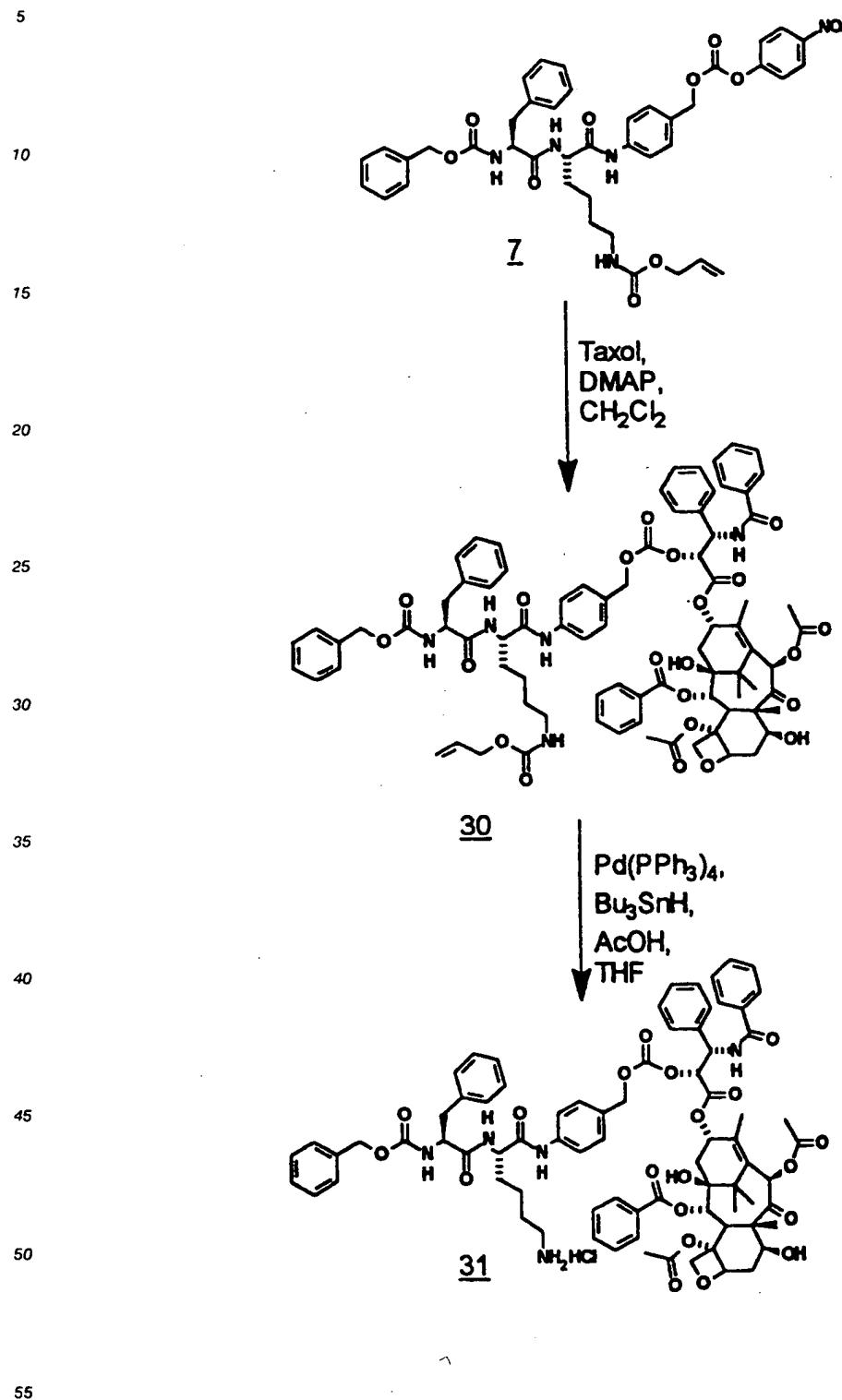


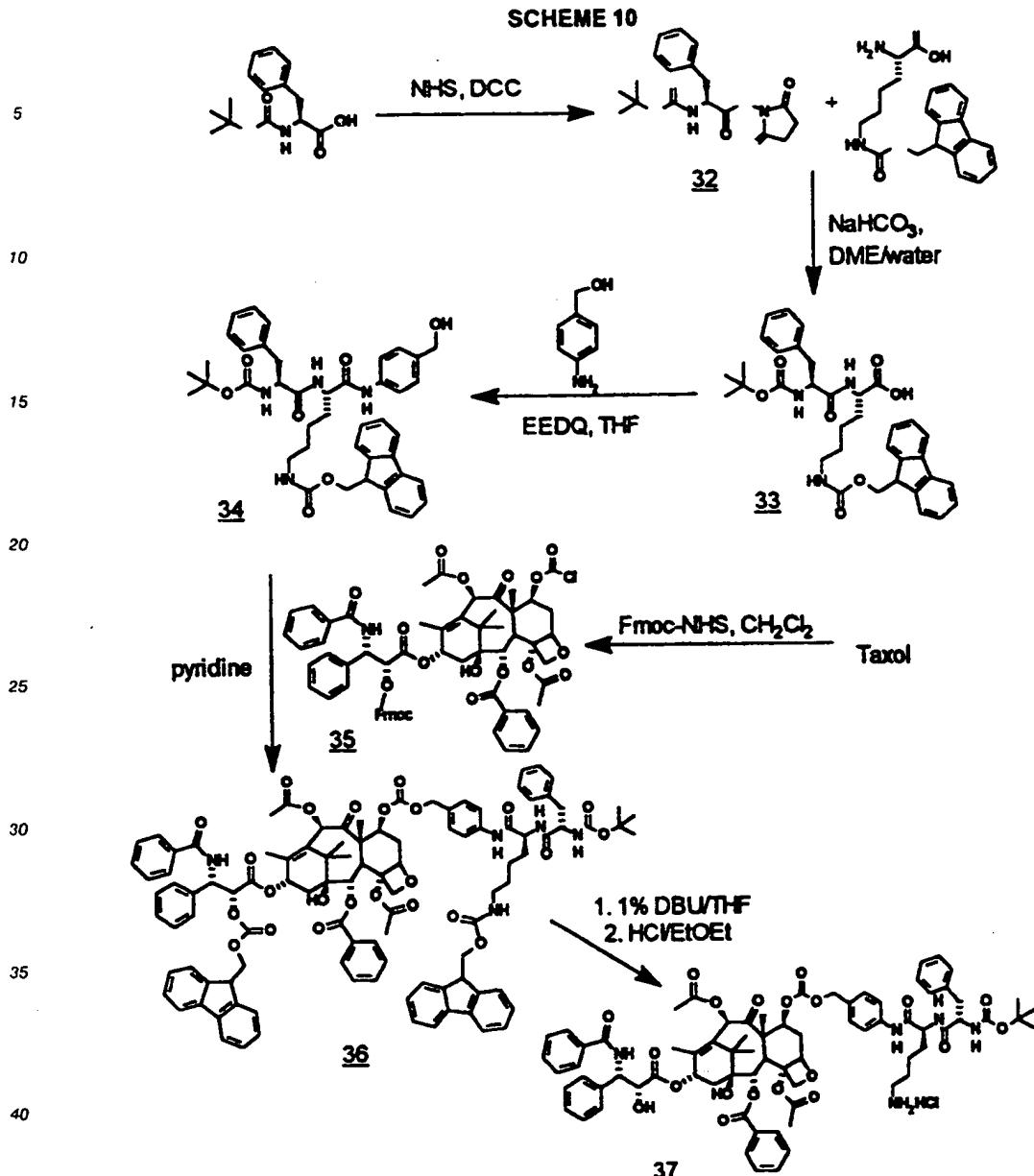
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SCHEME 9

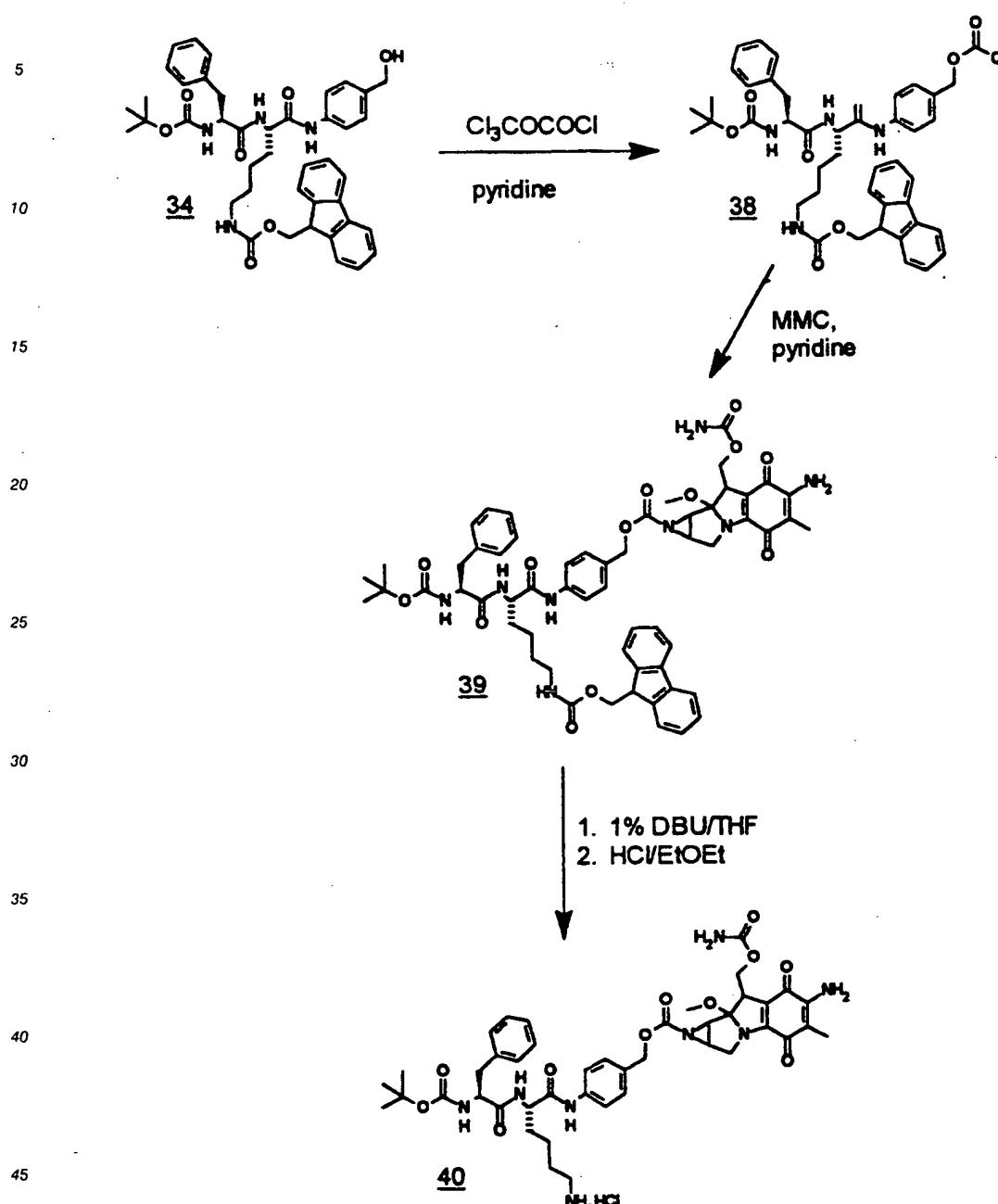




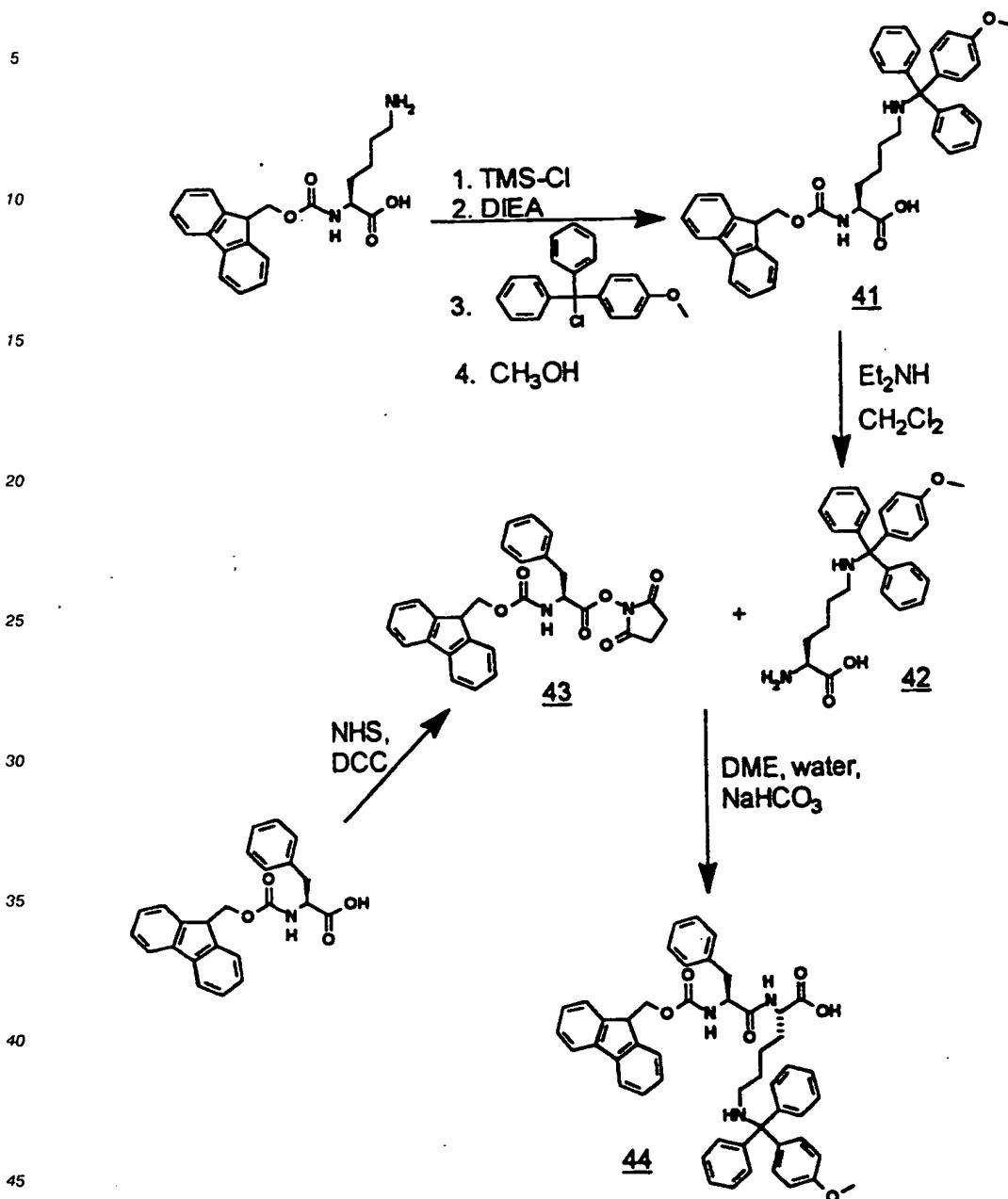
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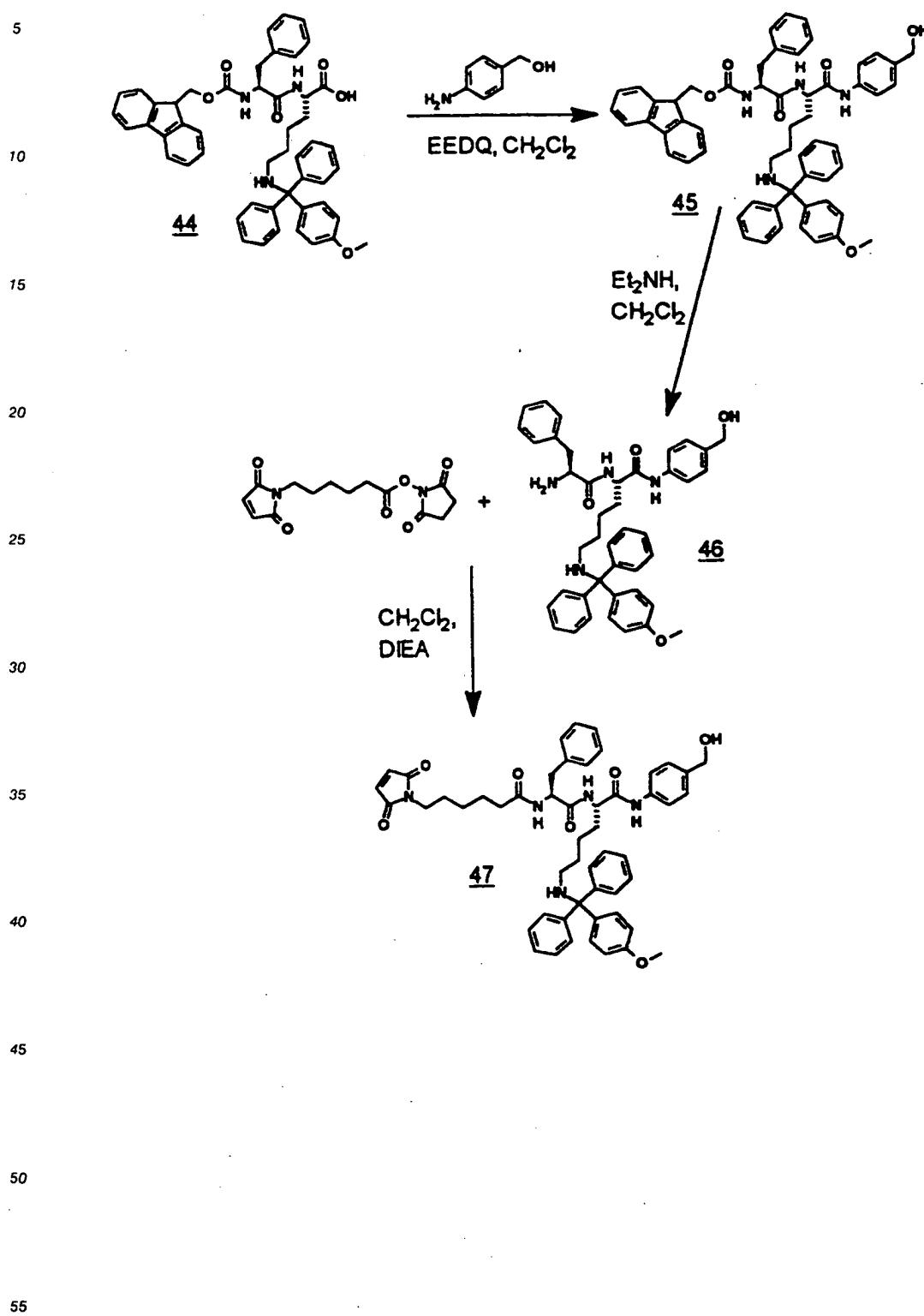
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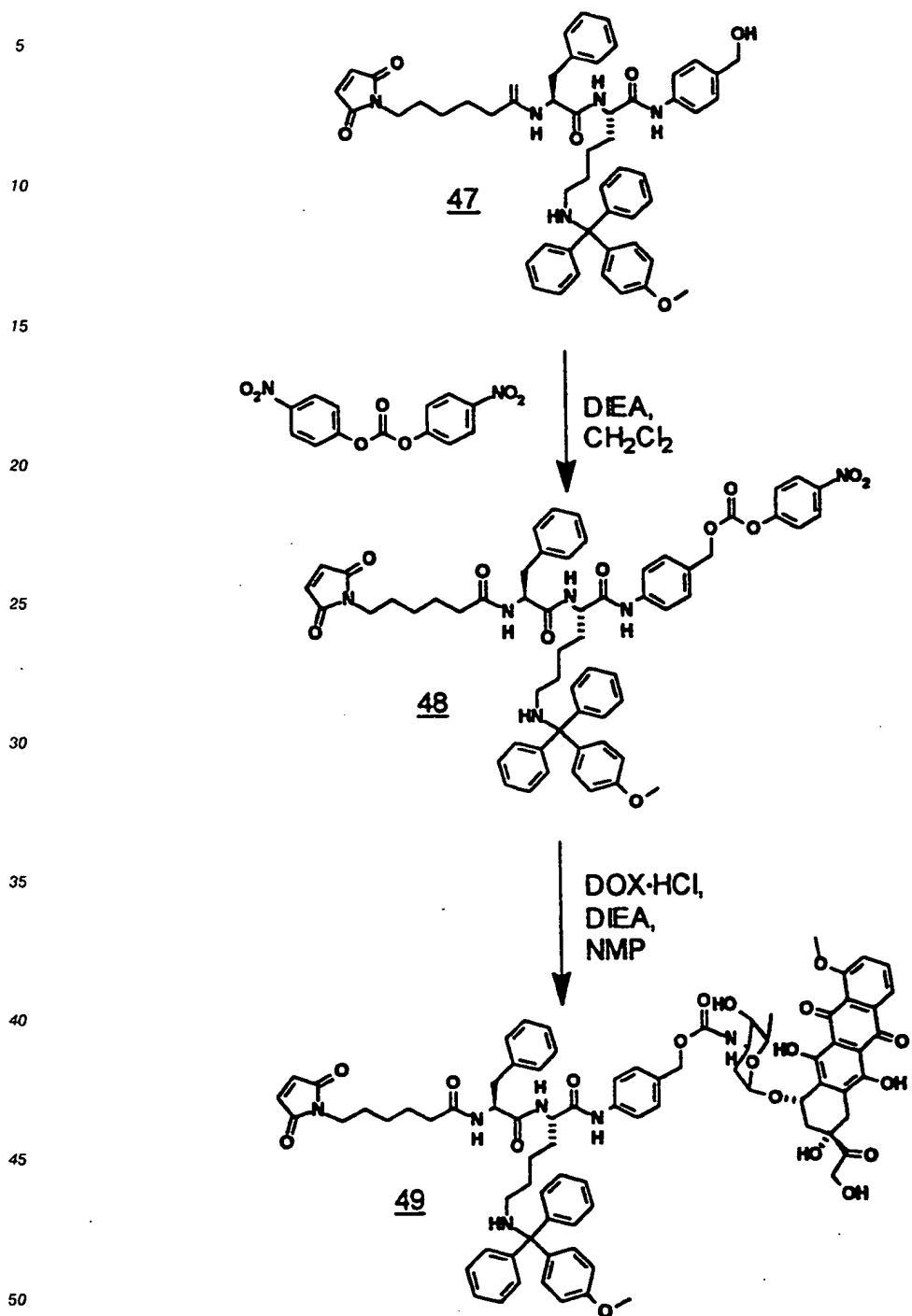
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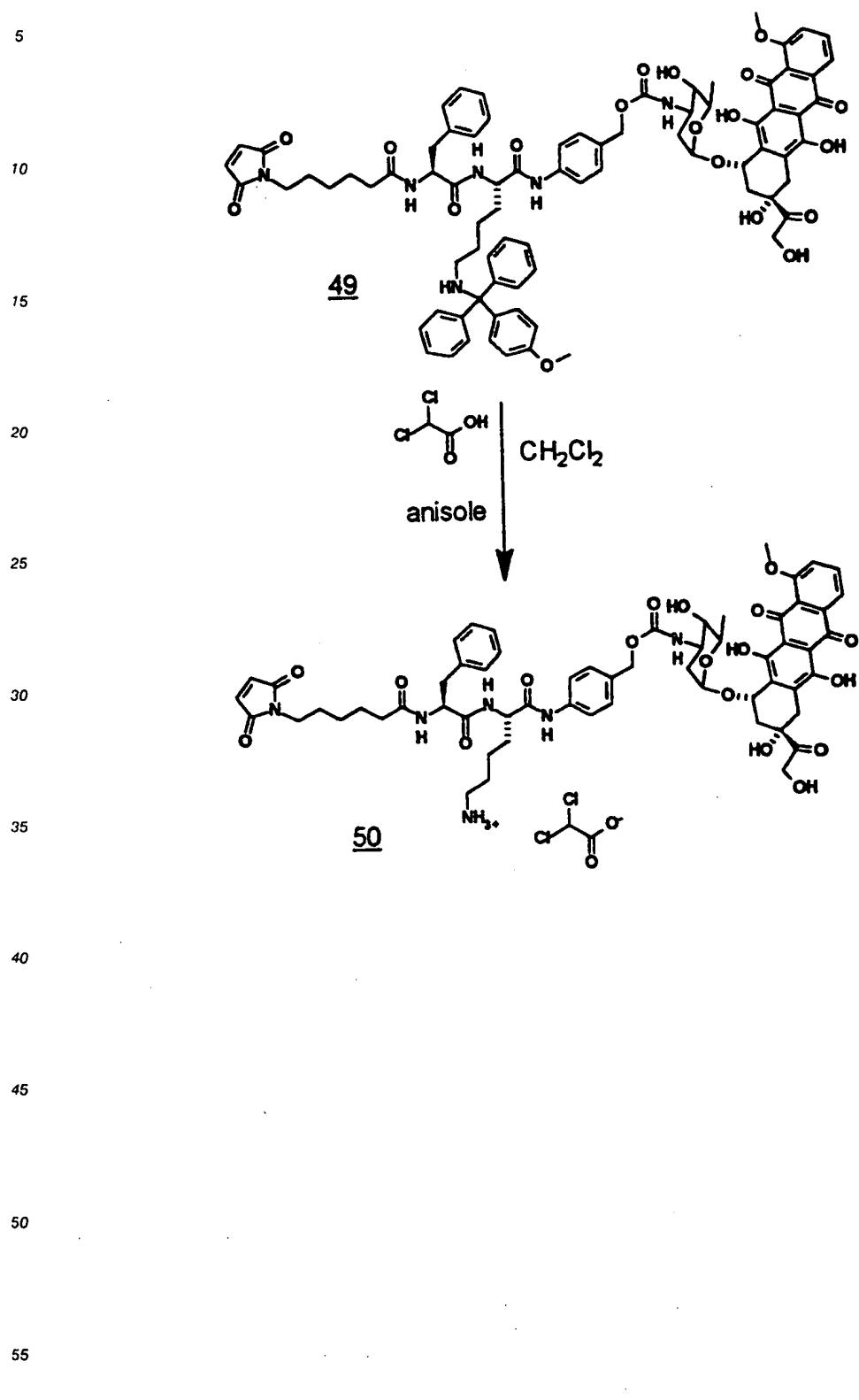
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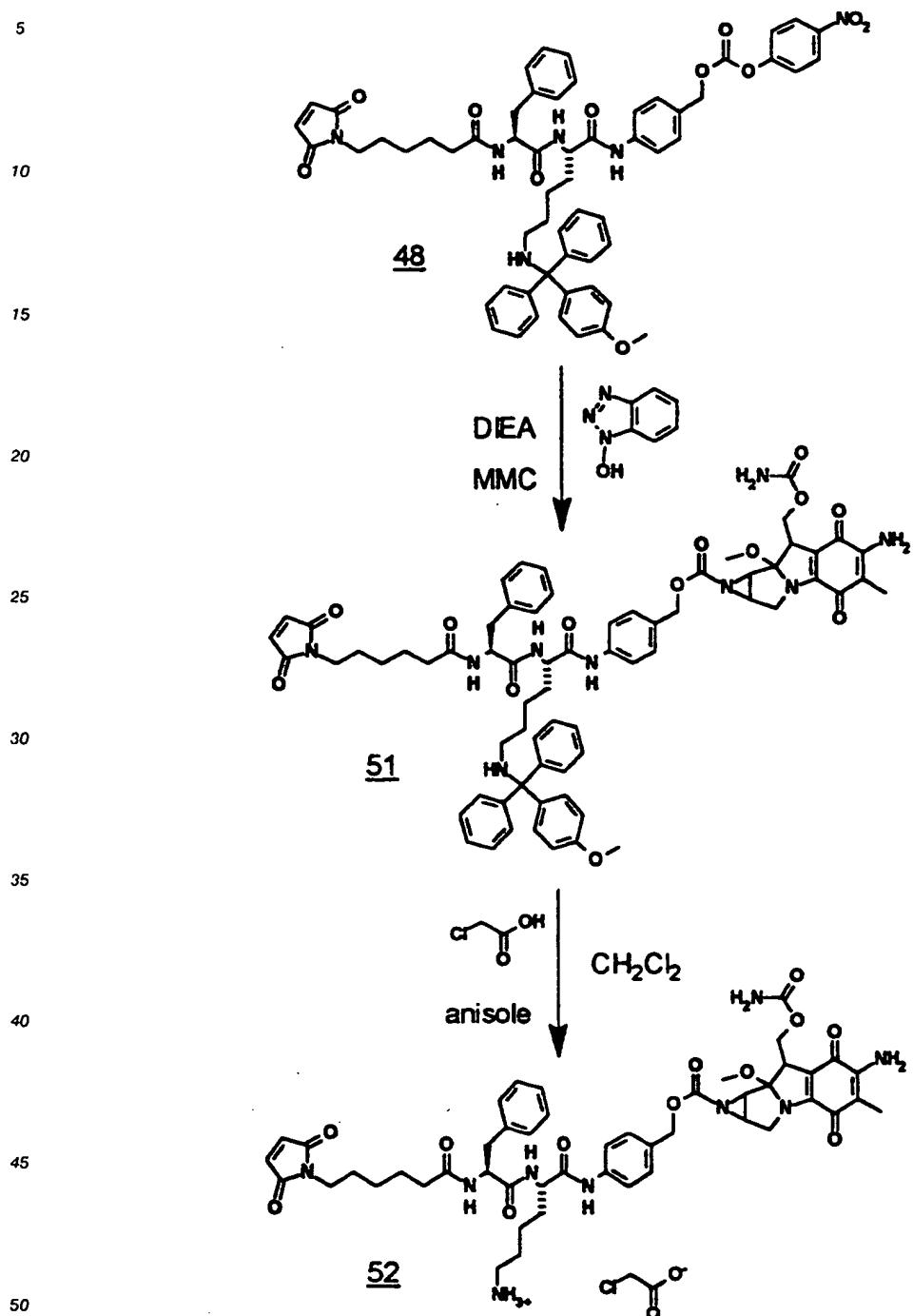
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SCHEME 12 - CONTINUED



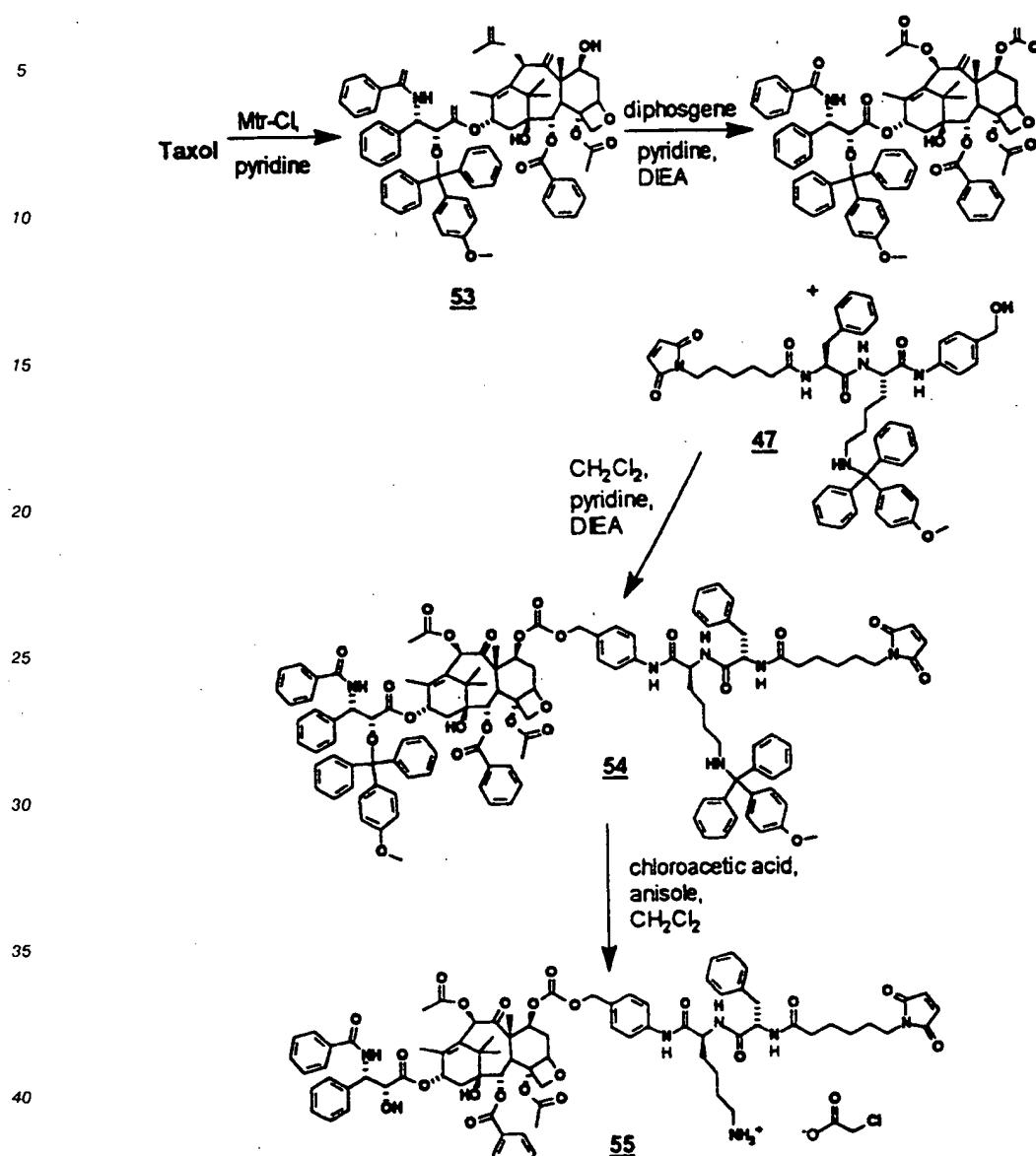
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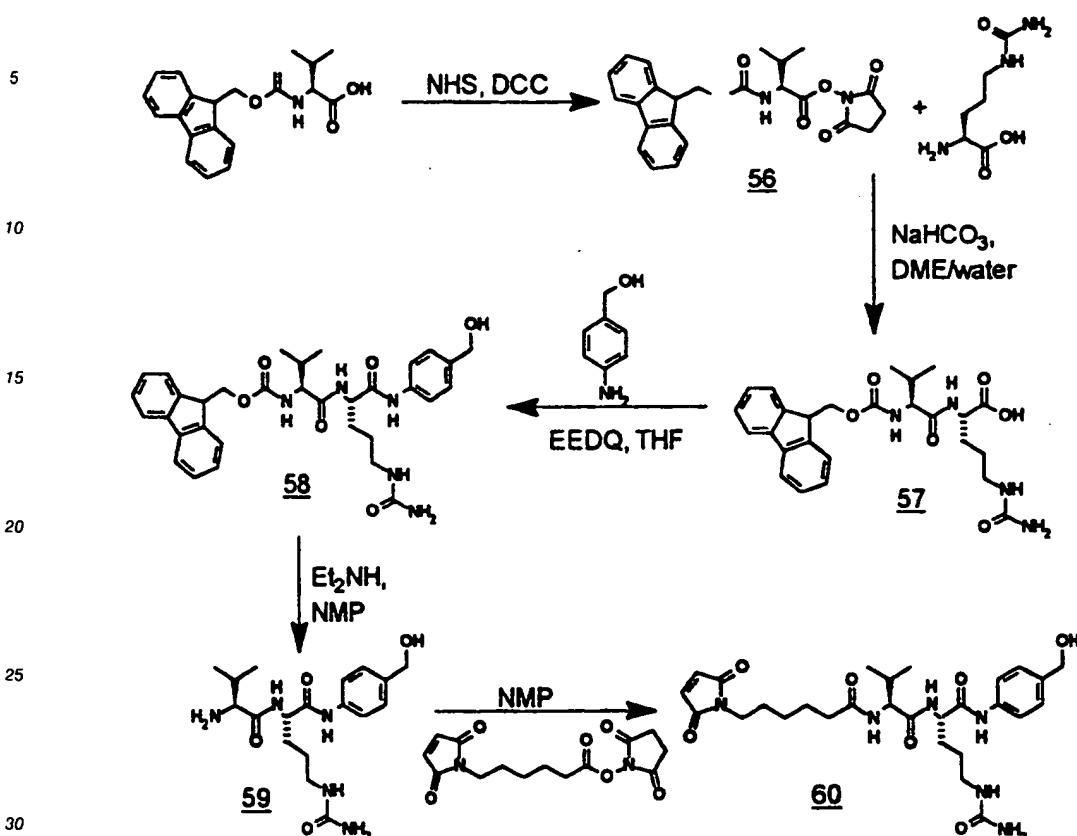
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SCHEME 14



SCHEME 15



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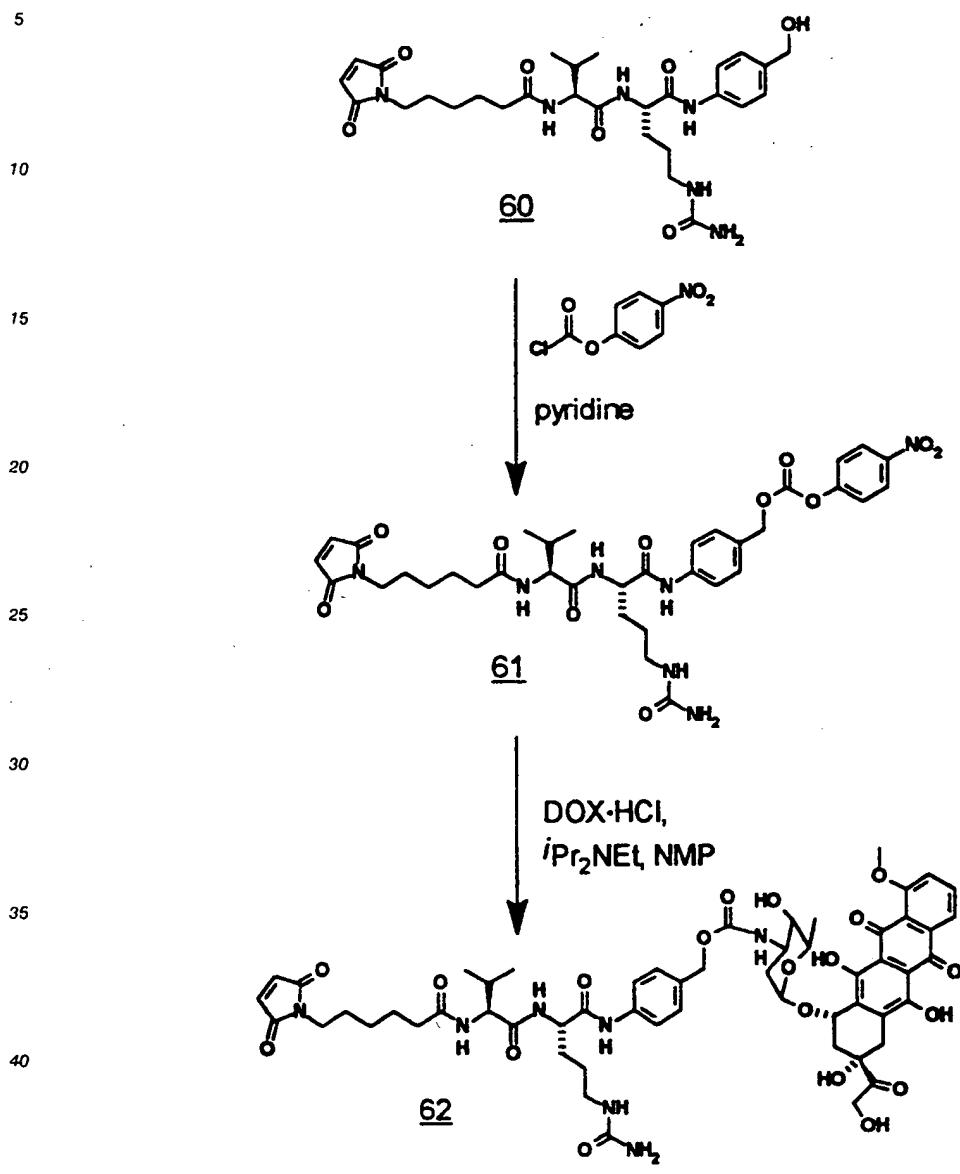
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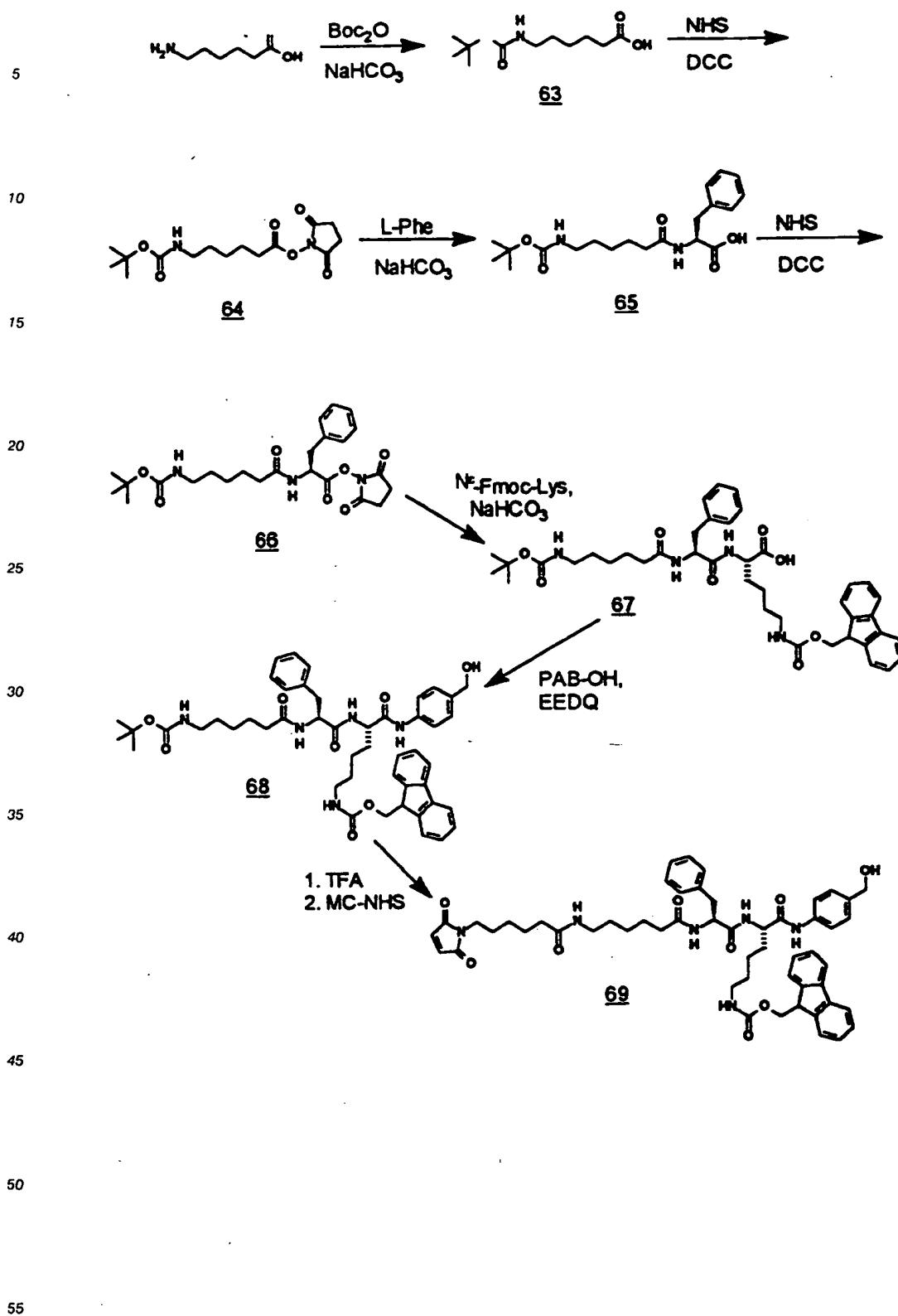
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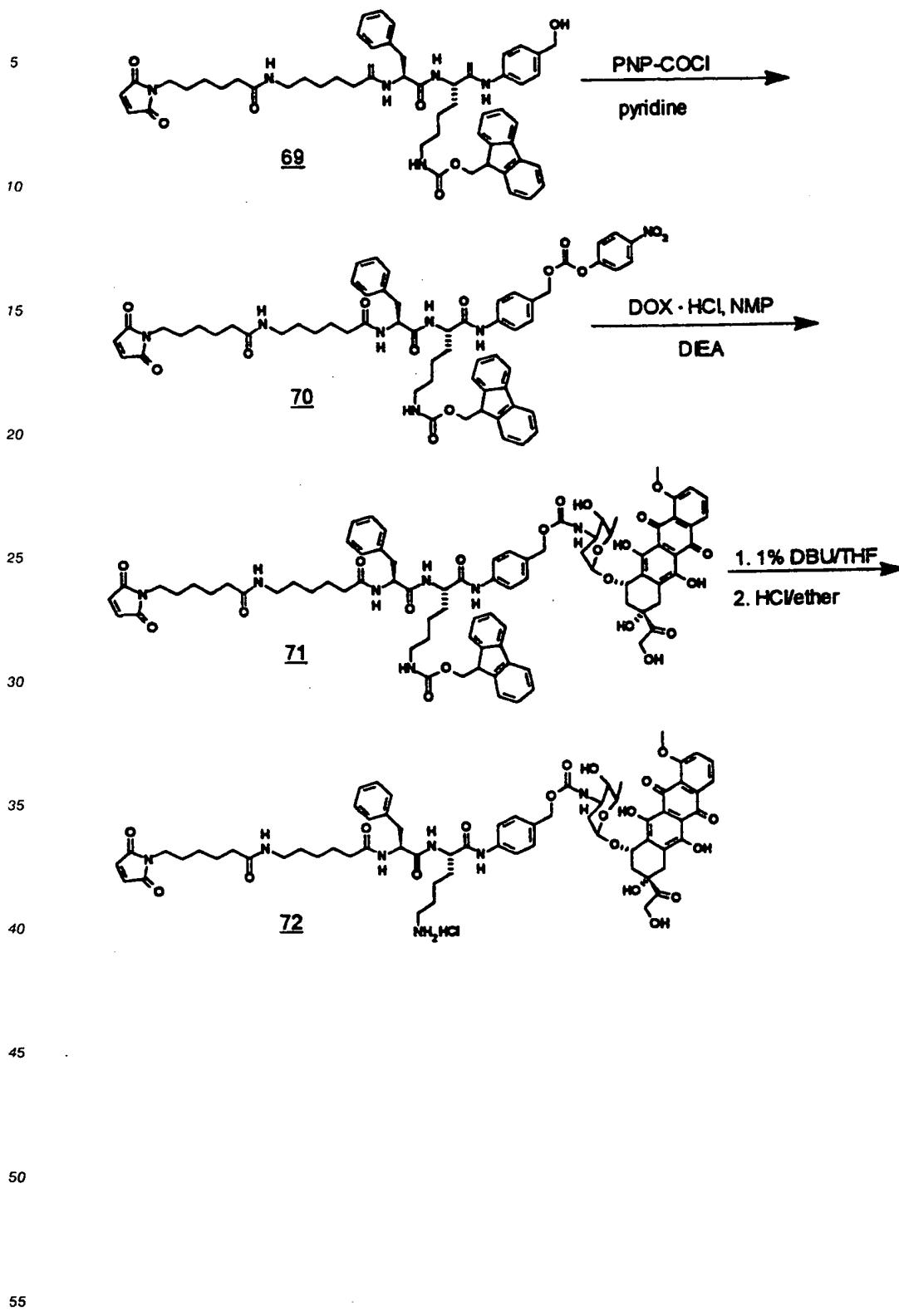
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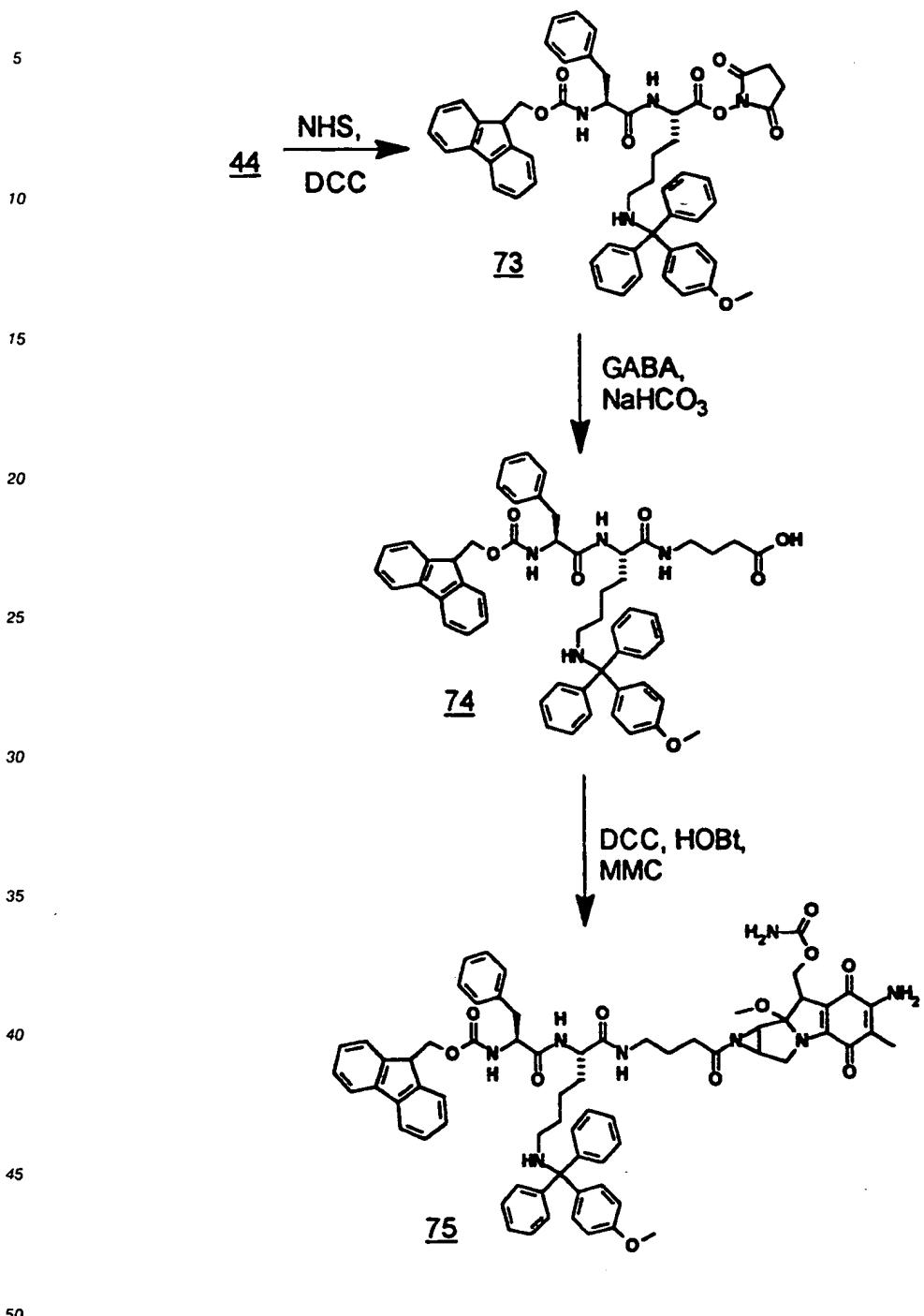
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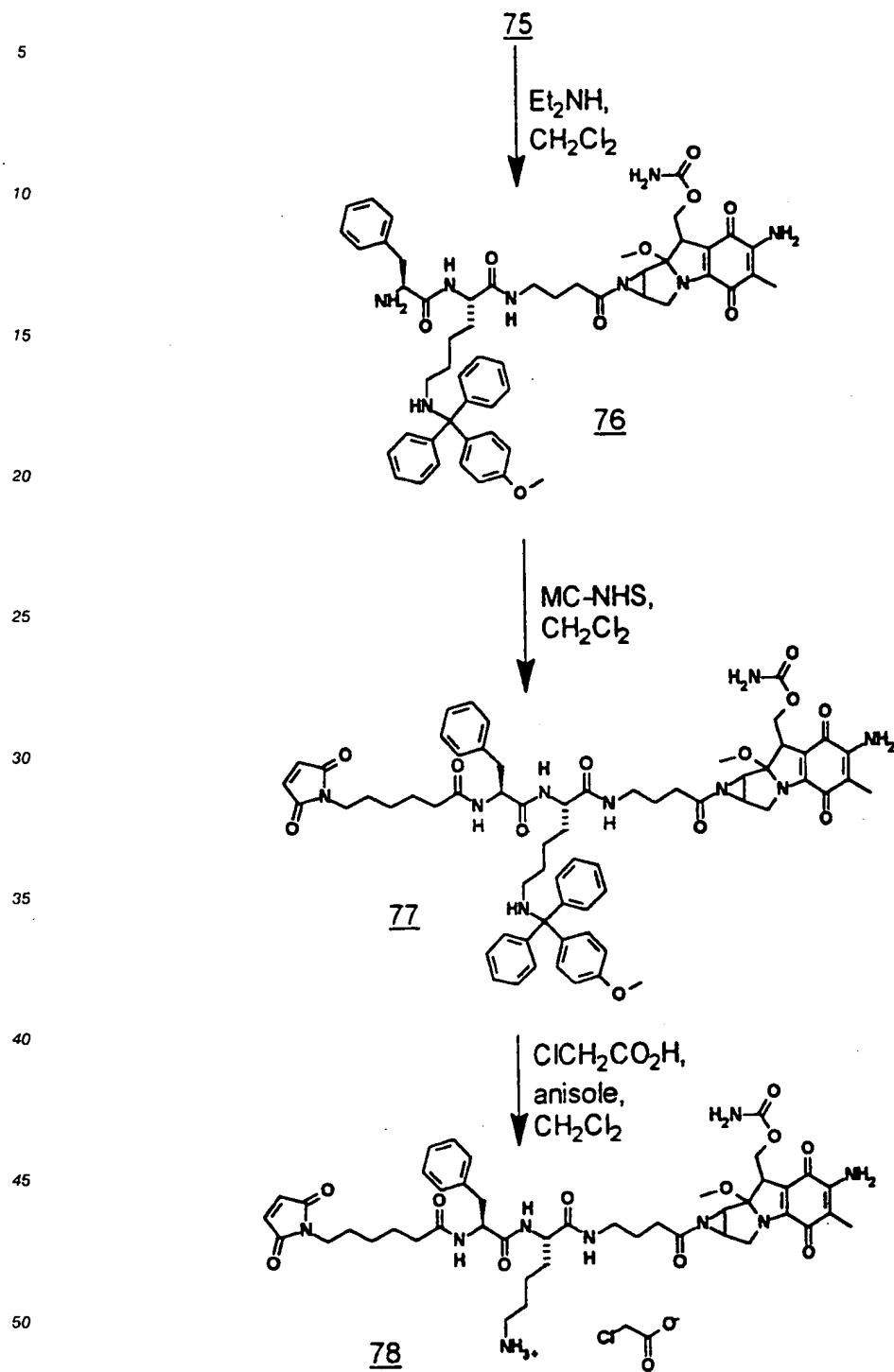
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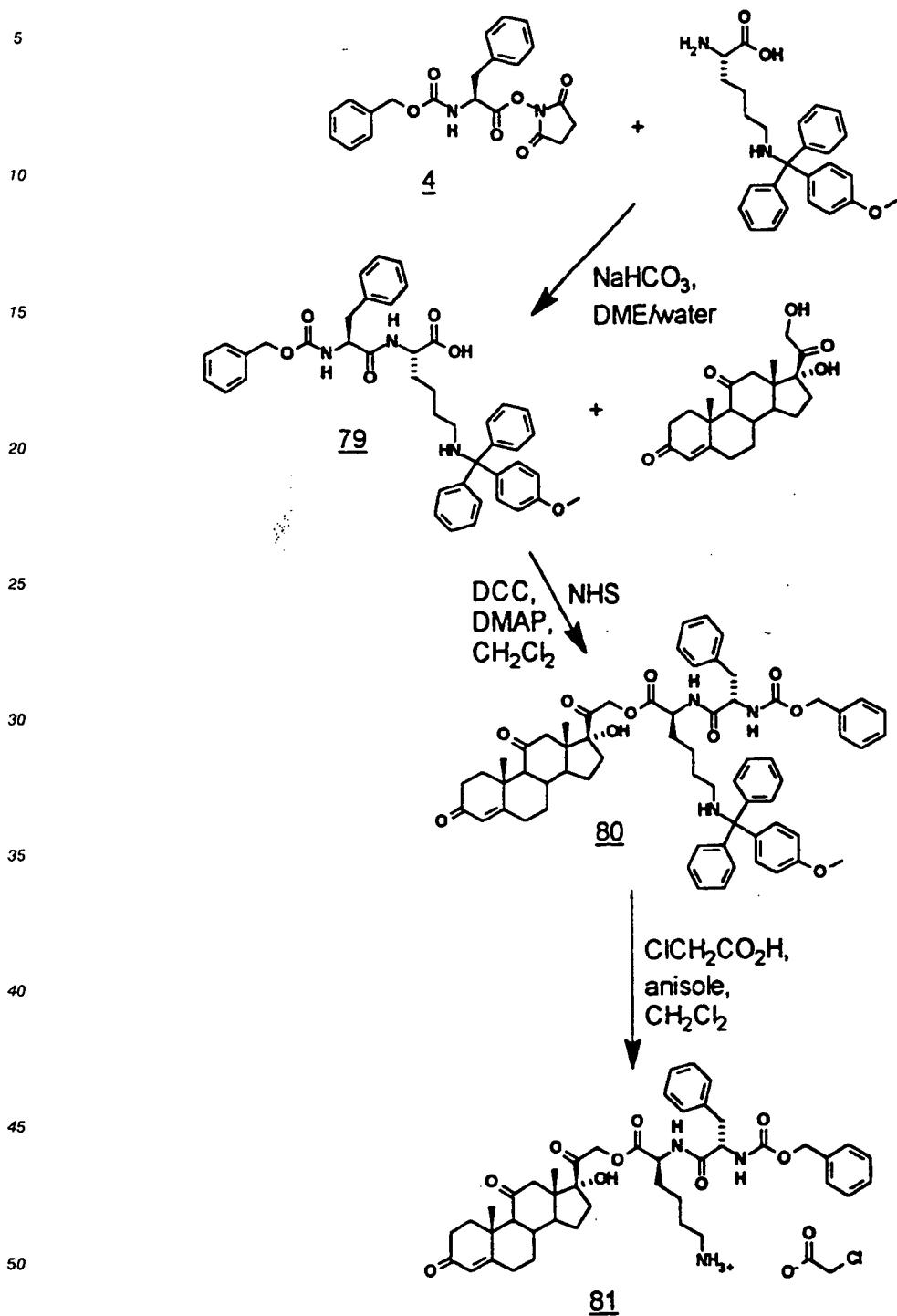
SCHEME 17



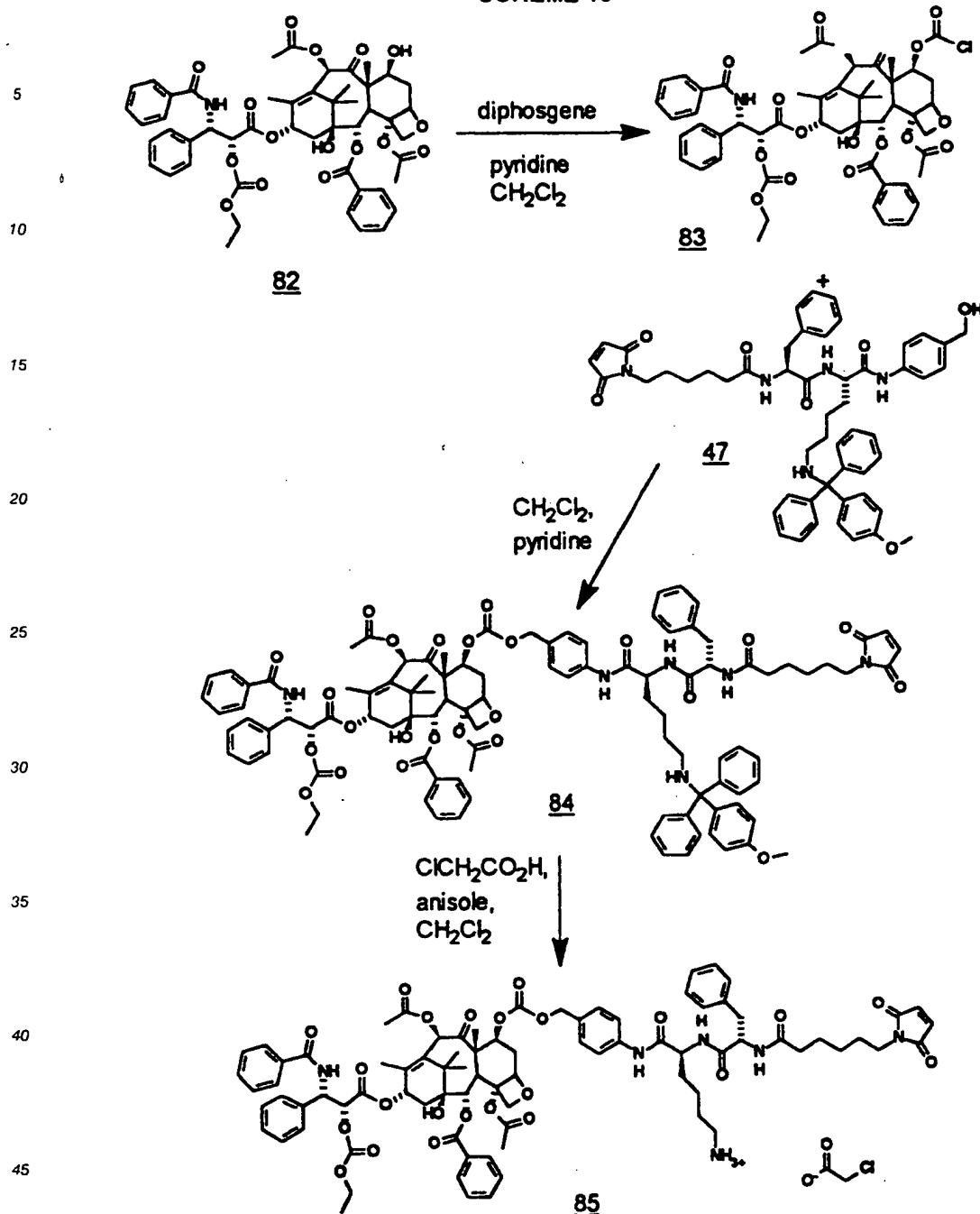
SCHEME 17 - CONTINUED



SCHEME 18



SCHEME 19



A preferred embodiment of the present invention refers to a method for preparation of maleimidocaproyl-phenylalanyl-lysine-p-aminobenzyl-carbamoyl-doxorubicin which comprises:

(a) treating a suspension of N^ε-9-fluorenylmethoxycarbamoyl-lysine in methylene chloride under argon at room temperature with trimethylsilyl chloride, heating the mixture at reflux for about one hour, cooling it to 0°C and adding diisopropylethylamine and p-anisyldiphenylmethyl chloride, to give N^ε-9-fluorenylmethoxycarbamoyl-N^ε-p-methoxytrityl-lysine after aqueous workup, extracting with ethyl acetate, and evaporating the solvent;

(b) removing the protecting group from N^ε-9-fluorenylmethoxycarbamoyl-N^ε-p-methoxytrityl-lysine by treating with a base like diethylamine in methylene chloride at room temperature for about 4 to 24 hours, followed by evaporating the solvent and triturating with ether to give N^ε-p-methoxytrityl-lysine;

(c) preparing 9-fluorenylmethoxycarbamoyl-phenylalanyl-N-hydroxysuccinimide by treating 9-fluorenylmethoxycarbamoyl-phenylalanine with dicyclohexylcarbodiimide and N-hydroxysuccinimide in methylene chloride at room temperature for about 12 to 24 hours, removing the dicyclohexylurea by-product by filtration and evaporating the solvent;

5 (d) coupling N^t-p-methoxytrityl-lysine and 9-fluorenylmethoxycarbamoyl-phenylalanyl-N-hydroxysuccinimide in a mixture of 1,2-dimethoxyethane and water in the presence of one equivalent of a base at room temperature for about 12 hours to 2 days, followed by acidifying the mixture and extracting the product, 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^t-p-methoxytrityl-lysine, with ethyl acetate;

10 (e) preparing 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^t-p-methoxytrityl-lysyl-p-aminobenzyl alcohol by treating 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^t-p-methoxytrityl-lysine with p-aminobenzyl alcohol and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline in methylene chloride at room temperature for about 12 to 24 hours, followed by evaporating the solvent and triturating with ether;

15 (f) removing the protecting group from 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^t-p-methoxytrityl-lysyl-p-aminobenzyl alcohol by treating with a base like diethylamine in methylene chloride at room temperature for about 4 to 16 hours, followed by evaporating the solvent and flash chromatography of the residue on silica, eluting with 2 to 4 percent methanol/methylene chloride to give phenylalanyl-N^t-p-methoxytrityl-lysyl-p-aminobenzyl alcohol;

20 (g) preparing maleimidocaproyl-phenylalanyl-N^t-p-methoxytrityl-lysyl-p-aminobenzyl alcohol by treating phenylalanyl-N^t-p-methoxytrityl-lysyl-p-aminobenzyl alcohol with maleimidocaproyl-N-hydroxysuccinimide in the presence of one equivalent of a base in methylene chloride, tetrahydrofuran or 1,2-dimethoxyethane for one to three days, followed by aqueous workup and triturating with ether;

25 (h) treating maleimidocaproyl-phenylalanyl-N^t-p-methoxytrityl-lysyl-p-aminobenzyl alcohol with three to five equivalents of bis-p-nitrophenyl carbonate and a base like diisopropylethylamine in methylene chloride at room temperature for two to five days, followed by aqueous workup and flash chromatography on silica gel, eluting with 1:1 to 8:1 ethyl acetate/hexane to give maleimidocaproyl-phenylalanyl-N^t-p-methoxytrityl-lysyl-p-aminobenzyl-p-nitrophenyl carbonate;

30 (i) coupling maleimidocaproyl-phenylalanyl-N^t-p-methoxytrityl-lysyl-p-aminobenzyl-p-nitrophenyl carbonate with doxorubicin in N-methylpyrrolidinone at room temperature for two to four days, followed by aqueous workup and flash chromatography on silica gel, and eluting with 25:1 to 20:1 methylene chloride/methanol to give maleimidocaproyl-phenylalanyl-N^t-p-methoxytrityl-lysyl-p-aminobenzylcarbamoyl-doxorubicin; and

35 (j) removing the lysine protecting group by treating with an acid (HA) in methylene chloride in the presence of a cation scavenger at room temperature for one to six hours, followed by precipitating the product, maleimidocaproyl-phenylalanyl-lysyl-p-aminobenzylcarbamoyl-doxorubicin•dichloro acetic acid by addition of ethyl acetate or ether and collecting by filtration.

40 Preferably the deprotection of maleimidocaproyl-phenylalanine-N^t-p-methoxytrityl-lysyl-p-aminobenzylcarbamoyl-doxorubicin comprises treating with ten equivalents of dichloroacetic acid and 100 equivalents of anisole in methylene chloride for one to three hours, followed by precipitating the product, maleimidocaproyl-phenylalanine-lysyl-p-aminobenzylcarbamoyl-doxorubicin•dichloroacetic acid with ethyl acetate.

Biological Activity

45 Representative conjugates of the present invention were tested in both in vitro and in vivo systems to determine biological activity. In these tests, the potency of conjugates of cytotoxic drugs was determined by measuring the cytotoxicity of the conjugates against cells of human cancer origin. The following describes representative tests used and the results obtained. One skilled in the art will recognize that any tumor line expressing the desired antigen could be used in substitution of the specific tumor lines used in the following analyses.

50 **TEST I**

Cathepsin B Release of Free DOX.

55 300 µl of the above conjugate solution was diluted to 1 ml with pH 5.0 acetate buffer (25 mM + 1 mM EDTA) giving a final pH of 5.3. This solution was incubated at about 37 °C while 6 µl of cathepsin B solution (see 2 below) was incubated with 20 µl of activating solution (see 2 below) for about 15 minutes at room temperature. The enzyme solution was then treated with the pH 5.3 conjugate solution and the mixture

incubated at about 37 °C. 25 µl aliquots were removed periodically and diluted with 50 µl of cold methanol to precipitate the protein. The samples were centrifuged and the liquid injected into the HPLC (C-18 column; 80:20 methanol/pH 2.8 triethylammonium formate buffer; 1 ml/min.; 495 nm detection wavelength). Peak areas were calibrated by injection of known concentration of DOX. The half-life of release of free DOX was determined to be about 3 hours with 93% of the theoretical release of DOX accounted for (some free DOX is likely to precipitate out with the protein).

TEST II

10 Human Plasma Stability.

300 µl of conjugate solution was diluted to 1 ml with freshly drawn human plasma and the mixture was incubated at about 37 °C. 25 µl aliquots were removed periodically and diluted with 50 µl of cold methanol. The samples were centrifuged and the liquid injected into the HPLC (conditions as above). Separate plasma samples were incubated with 1% and 2% theoretical release of free DOX for several minutes and treated in the same way. Free DOX was successfully detected and quantified at these levels. No free DOX was detected from the conjugate in plasma over 7.5 hours. (half-life > 375 hrs.).

TEST III

20 Cathepsin B Unmasking of Z-Phe-Lys-PABC-DOX

Bovin spleen cathepsin B (Sigma, EC 3.4.22.1, MW ca. 40,000) (10 units) was dissolved in 1 ml pH 5.0 acetate buffer (25mM acetate + 1mM EDTA), giving a solution roughly 13.7 M. 6 µl of the enzyme solution was incubated with 12 µl of an activating solution (30 mM dithiothreitol and 15 mM EDTA) for about 15 minutes at room temperature. To this was added 2 ml of pH 5.0 acetate buffer (25mM acetate with 1mM EDTA) which had been incubated at about 37 °C, followed by 8 µl of a 10 mM solution of Z-Phe-Lys-PABC-DOX in methanol ([Substrate] = 40 µM, [Cathepsin B] = ca. 41 nM). The mixture was incubated at about 37 °C, and aliquots were periodically removed and injected into the HPLC (C-18 column; 80:20 methanol/pH 2.8 triethylammonium formate (50mM) buffer; 1 ml/min.; 495 nm detection wavelength). The half-life of release of free DOX was determined to be 7-9 minutes.

TEST IV

35 Human plasma stability

4 µl of a 10mM solution of Z-Phe-Lys-PABC-DOX was dissolved in 1 ml of freshly drawn human plasma. Aliquots (50 µl) were periodically removed and diluted with cold methanol (100 µl). The samples were centrifuged and the resulting liquid injected into the HPLC (conditions as above). Enough DOX was added to a separate sample of plasma to give a theoretical release of 1% from the substrate. This was successfully detected using the same methods. No free DOX was detected from Z-Phe-Lys-PABC-DOX in plasma over 7 hours (half-life > 350 hrs.)

TEST V

45 Materials and Methods

Human Tumor Cell Lines. L2987 is a lung adenocarcinoma line obtained from I. Hellstrom (Bristol-Myers Squibb, Seattle, WA). The HCT116 colorectal tumor line was obtained from M. Brattain (Baylor Inst., TX).

50 A2780 is an ovarian carcinoma line obtained from K. Scanlon (National Cancer Institute).

Binding Assays. Binding assays were performed by indirect immunofluorescence. Briefly, target cells were harvested in logarithmic phase using trypsin/EDTA (GIBCO, Grand Island, NY) in PBS. The cells were washed twice in PBS containing 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) and resuspended to 1×10^7 /ml in PBS containing 1% BSA and 0.1% NaN₃. Cells (0.1 ml) were mixed with various antibodies (0.1 ml at 40 µg MAb/ml) and incubated for about 45 minutes at about 4 °C. The cells were washed 2x and resuspended in 0.1 ml of an appropriate concentration of rabbit anti-human IgG (Cappel Laboratories, Cochranville, PA, Fab'2 fragment). Cells were incubated for about 30 minutes at about 4 °C, washed 2x and kept on ice until analyzed on a Coulter EPICS 753 fluorescence-activated cell sorter.

Data are expressed as fluorescence intensity (FI): the mean channel number of specific minus control antibody.

In vitro cytotoxicity assays. Monolayer cultures of human carcinoma cells were harvested using trypsin-EDTA (GIBCO, Grand Island, NY), and the cells counted and resuspended to 1×10^5 /ml in RPMI-1640 containing 10% heat inactivated fetal calf serum (RPMI-10%FCS). Cells (0.1 ml/well) were added to each well of 96 well microtiter plates and incubated overnight at about 37°C in a humidified atmosphere of 5% CO₂. Media was removed from the plates and serial dilutions of DOX or MAb-DOX conjugates added to the wells. All dilutions were performed in quadruplicate. Cells were exposed to DOX or MAb-DOX conjugates for about 2 hours at about 37°C in a humidified atmosphere of 5% CO₂. Plates were then centrifuged (200 x g, 5 min.), the drug or conjugate removed, and the cells washed 3x with RPMI-10%FCS. The cells were cultured in RPMI-10%FCS (37°C, 5% CO₂) for an additional 48 hours. At this time the cells were pulsed for about 2 hours with 1.0 uCi/well of ³H-thymidine (New England Nuclear, Boston, MA). The cells were harvested onto glass fibre mats (Skatron Instruments, Inc., Sterling, VA), dried, and filter bound ³H radioactivity determined (β -Plate scintillation counter, Pharmacia LKB Biotechnology, Piscataway, NJ). Inhibition of ³H-thymidine uptake was determined by comparing the mean CPM for treated samples with that of the mean CPM of the untreated control.

Results

Binding Assays: The L2987, A2780 and HCT116 human carcinoma lines were evaluated for the expression of the BR96 antigen using direct immunofluorescence. As shown in Figure 1, the L2987 lung line expressed the greatest density of the BR96 antigen (FI = 172.8), the A2780 ovarian line expressed BR96 at a lower density (FI = 103.2), and the HCT116 colon line did not express significant amounts of the BR96 antigen (FI = 0).

Cytotoxicity of BR96-DOX peptide linked conjugate: The *in vitro* potency of the BR96-DOX peptide immunoconjugate was evaluated in parallel against the L2987, A2780, and HCT116 human carcinoma lines. As described above these cells express various densities of the BR96 antigen (L2987>A2780>>HCT116). Unconjugated doxorubicin was also evaluated. As shown in Figure 2, the potency of the BR96-DOX conjugate was equivalent to that of unconjugated DOX against the L2987 lung line. The BR96-DOX conjugate was approximately 50 fold less potent than unconjugated DOX against the A2780 ovarian line. The BR96-DOX conjugate was not active against the antigen-negative HCT116 line. However, as shown this line was sensitive to unconjugated DOX. These data demonstrate the direct relationship between the *in vitro* potency of the BR96-DOX conjugate and the epitope density of the BR96 antigen. In summary the BR96-DOX conjugate demonstrates antigen-specific cytotoxicity *in vitro* and the potency of the conjugate is related to the density of BR96 antigen expressed by various cell lines.

TEST VI

The BR96-PEP-DOX conjugate (MR = 4.41) was evaluated *in vivo* (Table 1) against L2987 human lung carcinoma xenografts. Therapy was initiated 14 days after tumor implant when the tumors were approximately 75 mm³ in size.

The BR96-PEP-DOX conjugate was active and tolerated at doses of 1.25-20 mg/kg equivalent DOX/injection. Higher doses were not evaluated in this first experiment. As shown in Table 1 the BR96-PEP-DOX conjugate was significantly more active than optimized DOX at doses of ≥ 2.5 mg/kg equivalent DOX/injection. The activity of the BR96-PEP-DOX conjugate administered at 1.25 mg/kg was similar to that of unconjugated DOX administered at 8 mg/kg. These data suggest that the *in vivo* potency of the BR96-PEP-DOX conjugates is similar to that of BMS-182248. The peptide-DOX conjugates will be evaluated for antigen-specific antitumor activity as soon as a non-binding (IgG-PEP-DOX) conjugate can be prepared.

50

55

Table 1

Antitumor activity of BR96-DOX peptide conjugates against established L2987 human tumor xenografts							
	Dose/Injection (mg/kg)		% Tumor Regressions				
	Treatment	DOX	BR96	Log Cell Kill	Complete	Partial	Number of Mice
5	DOX	8	-	2.4	10	0	10
		6	-	1.5	0	0	10
	BR96-DOX	20	1250	> 7	100	0	9
		10	625	> 7	89	11	10
		5	312	> 7	100	0	10
		2.5	156	> 7	90	10	10
		1.25	78	2.4	10	10	10
		0.63	39	0.3	0	0	10
		0.31	20	0.2	0	0	10

20 As a result of the above tests it can be seen that the compounds of the present invention are highly effective antitumor agents. They kill tumor cells *in vitro* via a specific targeting mechanism, in which the attached MAbs BR96 is the targeting moiety, as shown by the fact that cells which express high levels of the antigen recognized by the MAbs are efficiently killed; cells with less antigen are less efficiently killed; and cells without the antigen are not killed. Since all three cell types are sensitive to DOX, these results must arise from release of DOX after differential binding to the cells, not from differential toxicity of DOX to the various cell lines. The mechanism of the present invention is supported by the finding that Cathepsin B, a lysosomal protease, releases free DOX rapidly from both the peptide linker and the complete immunoconjugate. Since adventitious proteases in human blood do not release DOX from either the peptide linker or the complete immunoconjugate, it can be inferred that the immunoconjugate will reach tumor cells in human intact, without releasing free DOX enroute. Finally, *in vivo* experiments in tumor-bearing mice show that the immunoconjugate of the present invention produces remissions of antigen-positive tumors, with greater potency and less toxicity to the host than free DOX.

25 Thus, in an embodiment of the present invention, there is provided a method for the treatment of a neoplastic disease which comprises administering to a warm-blooded animal in need thereof, a therapeutically effective or biological function modifying amount of a conjugate of Formula (I). As can be appreciated, the particular conjugate used will depend on the disease state to be treated or the biological system to be modified. In particular, one skilled in the art will be able to select a particular ligand and drug to prepare a conjugate of Formula (I) which has specificity for the treatment of the disease or is able to modify the biological function desired.

30 A particularly preferred conjugate for this purpose is an immunoconjugate in which the drug moiety is doxorubicin and the ligand portion is selected from the group consisting of BR96, chimeric BR96, and the antigen-recognizing fragments thereof. The most preferred ligand for this embodiment is chimeric BR96, and the antigen-recognizing fragments thereof.

35 In a further embodiment, there is provided a process for preparing a compound of Formula (I), as previously defined.

40 The conjugates of the invention are administered to the patient in the form of a pharmaceutical formulation which comprises a conjugate of Formula (I) and a pharmaceutically acceptable carrier, excipient or diluent therefor. As used, "pharmaceutically acceptable" refers to those agents which are useful in the treatment or diagnosis of a warm-blooded animal including, for example, a human, equine, porcine, bovine, murine, canine, feline, or other mammal, as well as an avian or other warm-blooded animal. The preferred mode of administration is parenterally, particularly by the intravenous, intramuscular, subcutaneous, intraperitoneal, or intralymphatic route. Such formulations can be prepared using carriers, diluents or excipients familiar to one skilled in the art. In this regard, See, e.g. Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Company, edited by Osol et al. Such compositions may include proteins, such as serum proteins, for example, human serum albumin, buffers or buffering substances such as phosphates, other salts, or electrolytes, and the like. Suitable diluents may include, for example, sterile water, isotonic saline, dilute aqueous dextrose, a polyhydric alcohol or mixtures of such alcohols, for example, glycerin, propylene glycol, polyethylenglycol and the like. The formulations may contain

preservatives such as phenethyl alcohol, methyl and propyl parabens, thimerosal, and the like. If desired, the formulation can include 0.05 to about .20 percent by weight of an antioxidant such as sodium metabisulfite or sodium bisulfite.

For intravenous administration, the formulation preferably will be prepared so that the amount administered to the patient will be from about 1 to about 250 g of the desired conjugate. Preferably, the amount administered will be in the range of about 4 g to about 25 g of the conjugate. The conjugates of the invention are effective over a wide dosage range depending on factors such as the disease state to be treated or the biological effect to be modified, the manner in which the conjugate is administered, the age, weight and condition of the patient as well as other factors to be determined by the treating physician.

10 Thus, the amount administered to any given patient must be determined on an individual basis.

All publications cited in this specification are indicative of the level of skill of those in the art to which this application pertains. Each publication is individually incorporated herein by reference in the location where it is cited.

One skilled in the art will appreciate that although specific reagents and reaction conditions are outlined in the following Preparations and Examples, modifications can be made which are meant to be encompassed by the spirit and scope of the invention. The following Preparations and Examples, therefore, are provided to further illustrate the invention.

EXAMPLE 1

20 Preparation of Allyl-p-nitrophenyl carbonate (1)

Allyl alcohol (0.5 ml, 7.35 mmoles) in CH_2Cl_2 (3 ml) at room temperature was treated with p-nitrophenyl chloroformate (1.482 g, 1 equiv.). To this was added pyridine (0.6 ml, 1 equiv.) in CH_2Cl_2 (2 ml), dropwise over 10 minutes. After about 5 hours at room temperature the mixture was washed with 15% citric acid, water and brine, dried, and evaporated to give a thick, pale yellow oil. This was chromatographed on silica, eluting with 10-50% EtOAc/hexane, to give the product as an off-white, crystalline solid (1.542 g, 94%). $^1\text{H-NMR}$ (CDCl_3): δ 4.78 (2H, d, $\text{CH}_2\text{-O}$), 5.40 (2H, q, vinyl CH_2), 5.99 (1H, m, vinyl CH), 7.37 and 8.26 (4H, 2 x d, Ph); MS (DCI): 224 (MH^+);

30

Anal. calc. for $\text{C}_{10}\text{H}_9\text{NO}_5$:			
Found:	C-53.82, C-53.73,	H-4.06, H-4.03,	N-6.28; N-6.23.

35

EXAMPLE 2

40 Preparation of N^ε-Boc-N^ε-alloc-Lys (2)

A solution of Boc-Lys (8.4414 g, 34.27 mmoles) and NaHCO_3 (2.88 g, 1 equiv.) in water (50 ml) was added to allyl-p-nitrophenyl carbonate (1) (7.649 g, 1 equiv.) in DME (50 ml) at room temperature. The mixture was stirred overnight at room temperature. Water (80 ml) was then added and the mixture was extracted with ether (3 x 50 ml). The aqueous layer was acidified to pH 2 with 10% citric acid and then extracted with EtOAc (3 x 80 ml). The combined organic components were washed with water and brine, dried, and evaporated to give a white solid. This was treated with ether (100 ml) and the resulting mixture was sonicated for about 15 minutes to dissolve p-nitrophenol and then the solid (10.303 g, 91%) was collected by filtration and washed repeatedly with ether. $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$): δ 1.41 (9H, s, t-Bu), 1.49 and 1.70 (6H, m, Lys CH_2), 3.13 (2H, m, Lys N- CH_2), 4.25 (1H, m, CH), 4.52 (2H, d, allyl O- CH_2), 5.24 (2H, q, vinyl CH_2), 5.87 (1H, m, vinyl CH); MS (DCI): 331 (MH^+), 275 ($\text{MH}^+ \cdot \text{C}_4\text{H}_8$).

EXAMPLE 3

55 Preparation of N^ε-Alloc-Lys-TFA (3)

N^ε-Boc-N^ε-alloc-Lys 2 (9.94 g, 30 mmoles) in CH_2Cl_2 (50 ml) was treated with TFA (19 ml) at room temperature. The mixture was sonicated briefly and then stirred for about 1 hour. The solvents were evaporated at about 40 °C and the resulting yellow gum was triturated with ether (75 ml), giving a white

solid (8.58 g, 83%). $^1\text{H-NMR}$ (D_2O): δ 1.46 and 1.87 (4H and 2H resp., m, Lys CH_2), 3.11 (2H, m, N- CH_2), 3.80 (1H, t, Lys CH), 4.51 (2H, br s, allyl O- CH_2), 5.22 (2H, q, vinyl CH_2), 5.90 (1H, m, vinyl CH); MS (DCI): 231 (MH^+);

5

<u>Anal. calc. for $\text{C}_{12}\text{H}_{19}\text{N}_2\text{O}_6\text{F}_3$:</u>			
Found:	C-41.86, C-42.30,	H-5.56, H-5.52,	N-8.14; N-8.29.

10

EXAMPLE 4

Preparation of Z-Phe-NHS (4)

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Z-Phe (11.03 g, 36.85 mmoles), and NHS (4.45 g, 1.1 equiv.) in THF (50 ml) at about 0 °C were treated with DCC (7.98 g, 1.05 equiv.). After a few minutes a heavy white precipitate appeared. The mixture was allowed to warm to room temperature and was stirred for about 16 hours. The solid DCU by-product was filtered off and the filtrate was evaporated. The resulting thick, colorless oil was dissolved in CH_2Cl_2 (80 ml). The mixture was allowed to stand for an hour and was then filtered to remove more DCU. The filtrate was evaporated and the resulting colorless glass was dried in *vacuo* for about 3 hours, giving a foamy solid (14.023 g, 96%) that was used without further purification. $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$): δ 2.88 (4H, s, NHS CH_2), 3.27 (2H, m, Phe CH_2), 4.70 (1H, m, Phe CH), 5.13 (2H, s, Z CH_2), 7.27 (10H, m, Ph).

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EXAMPLE 5

Preparation of Z-Phe-N'-alloc-Lys (5)

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Z-Phe-NHS (4) (2.783 g, 7.021 mmoles) in DME (30 ml) at room temperature was treated with a solution of N'-alloc-Lys-TFA (2.54 g, 1.05 equiv.) and NaHCO_3 (1.24 g, 2.1 equiv.) in water (30 ml). The mixture was stirred vigorously at room temperature for 2 days. A small amount of DCU was removed by filtration and the filtrate was diluted with water (50 ml) and then acidified to pH 3 with 15% citric acid. The resulting mixture was extracted with EtOAc (3 x 80 ml) and the combined organic layers were washed with water and brine, dried, and evaporated to give a glassy solid. This was treated with ether (150 ml), sonicated, and heated in a water bath (50 °C). Upon cooling, the white solid product (2.79 g, 78%) was collected by filtration and washed with ether. $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$): δ 1.25, 1.43, 1.74 and 1.81 (6H, m, Lys CH_2), 3.00 (2H, m, Phe CH_2), 3.08 (2H, m, N- CH_2), 4.43 (2H, m, CO-CH), 4.48 (2H, d, allylic O- CH_2), 5.02 (2H, m, Z CH_2), 5.20 (2H, q, vinyl CH_2), 5.84 (1H, m, vinyl CH), 7.22 (10H, m, Ph); MS (FAB): 512 (MH^+), 534 ($\text{M} + \text{Na}^+$), 556 ($\text{M} + \text{K}^+$);

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<u>Anal. calc. for $\text{C}_{27}\text{H}_{33}\text{N}_3\text{O}_7$:</u>			
Found:	C-63.39, C-62.98,	H-6.50, H-6.48,	N-8.21; N-8.21.

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EXAMPLE 6

Preparation of Z-Phe-N'-alloc-Lys-PAB-OH (6)

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Z-Phe-N'-alloc-Lys (5) (524.7 mg, 1.026 mmoles) and p-aminobenzyl alcohol (133 mg, 1.05 equiv.) in THF (10 ml) at room temperature were treated with EEDQ (266.3 mg, 1.05 equiv.). The mixture was stirred at room temperature for about 16 hours. The mixture was evaporated to dryness at about 30 °C and the residue triturated with ether (15 ml). The resulting white solid product (591.6 mg, 94%) was collected by filtration and washed with ether. $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$): δ 1.25, 1.42, 1.59 and 1.77 (6H, m, Lys CH_2), 2.97 (2H, m, Ph CH_2), 3.06 (2H, m, N- CH_2), 4.37 (2H, m, Ph and Lys CH), 4.46 (2H, d, allyl O- CH_2), 4.55 (2H, s, Ph- $\text{CH}_2\text{-OH}$), 4.98 (2H, m, Z CH_2), 5.18 (2H, q, vinyl CH_2), 5.81 (1H, m, vinyl CH), 7.08 and 7.43 (4H, 2 x d, PAB Ph), 7.11 and 7.23 (10H, m, Z and Phe Ph); MS (FAB): 617 (MH^+), 639 ($\text{M} + \text{Na}^+$), 655 ($\text{M} + \text{K}^+$);

Anal. Calc. for C ₃₄ H ₄₀ N ₄ O ₇ :			
	C-66.22, C-65.72,	H-6.54, H-6.43,	N-9.08, N-8.92.
Found:			

5

EXAMPLE 710 Preparation of Z-Phe-N'-alloc-Lys-PABC-PNP (7)

Z-Phe-N'-alloc-Lys-PAB-OH (6) (269.6 mg, 437.2 μ moles) in dry THF (8 ml) at room temperature was treated with p-nitrophenyl chloroformate (106 mg, 1.2 equiv.) and pyridine (42.5 μ l, 1.2 equiv.). After about 6 hours TLC (silica; 25:1 CH₂Cl₂/CH₃OH) indicated completion. EtOAc (25 ml) and 10% citric acid (25 ml) were added. The organic layer was washed with water and brine, dried, and evaporated to give a yellow solid which was chromatographed on silica, eluting with 30:1 CH₂Cl₂/CH₃OH, to give the product as an off-white solid (297.4 mg, 87%). ¹H-NMR (CDCl₃/CD₃OD): δ 1.24, 1.42, 1.59 and 1.78 (6H, m, Lys CH₂), 2.97 (2H, m, N-CH₂), 3.04 (2H, m, Phe CH₂), 4.38 (2H, m, Phe and Lys CH), 4.46 (2H, d, allyl O-CH₂), 5.01 (2H, s, Z CH₂), 5.17 (2H, q, vinyl CH₂), 5.21 (2H, s, PAB CH₂-O), 5.37 and 5.80 (each 1H, m, Phe and Lys NH), 5.83 (1H, m, vinyl CH), 7.11 and 7.56 (4H, 2 x d, PAB Ph), 7.13 and 7.25 (10H, m, Phe and Z Ph), 7.35 and 8.10 (each 2H, d, PNP Ph), 9.23 (1H, br s, PAB NH); MS (FAB): 782 (M+H)⁺, 804 (M+Na)⁺, 820 (M+K)⁺;

15

Anal. calc. for C ₄₁ H ₄₃ N ₅ O ₁₁ :			
	C-62.99, C-62.75,	H-5.54, H-5.49,	N-8.96; N-8.86.
Found:			

20

EXAMPLE 8

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30 Preparation of Z-Phe-N'-alloc-Lys-PABC-DOX (8)

Z-Phe-N'-alloc-Lys-PABC-PNP (7) (337.2 mg, 431.3 μ moles) and DOX-HCl (275.2 mg, 1.1 equiv.) in NMP (8 ml) at room temperature were treated with triethylamine (66 μ l, 1.1 equiv.). The mixture was allowed to stand in the dark for about 2 days. The mixture was then diluted with 10% i-Pr-OH/EtOAc (100 ml) and washed with water (3 x 100 ml) and brine, dried, and evaporated to give an orange solid. This was chromatographed on silica, eluting with 1) 25:1 and 2) 15:1 CH₂Cl₂/CH₃OH, to give the product as an orange solid (496.3 mg, 97%). ¹H-NMR (CDCl₃/CD₃OD): δ 1.18 (3H, d, sugar CH₃), 1.22, 1.38, 1.56 and 1.77 (6H, m, Lys CH₂), 1.74 (2H, m, D-ring-CH₂), 2.23 (2H, m, D-ring CH₂), 2.95 (2H, m, sugar CH₂), 3.02 (2H, m, N-CH₂), 3.53 (1H, s, sugar HO-CH), 3.80 (1H, m, sugar HN-CH), 3.99 (3H, s, OCH₃), 4.06 (1H, m, sugar CH₃-CH), 4.39 (2H, m, Phe and Lys CH), 4.43 (2H, d, allyl O-CH₂), 4.70 (2H, s, PAB CH₂-O), 4.89 (2H, m, Z CH₂), 4.92 (1H, m, anomeric CH), 4.96 (2H, d, CO-CH₂-OH), 5.15 (2H, q, vinyl CH₂), 5.11, 5.39 (each 1H, s, OH), 5.41 (1H, br, DOX Ph-CH), 5.60 and 5.92 (each 1H, m, amide NH), 5.79 (1H, m, vinyl CH), 7.08 and 7.23 (10H, m, Phe and Z Ph), 7.13 and 7.40 (4H, 2 x d, PAB Ph), 7.50, 7.68 and 7.90 (each 1H, m, DOX Ph), 9.15 (1H, br s, PAB NH); MS (FAB): 1209 (M+Na)⁺, 1224 (M+K)⁺; HRMS (FAB): Accurate mass calc. for C₆₂H₆₇N₅O₁₉: 1186.4509; found: 1186.4463.

EXAMPLE 9

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Preparation of Z-Phe-Lys-PABC-DOX-HCl (9)

Z-Phe-N'-alloc-Lys-PABC-DOX (8) (34.9 mg, 29.4 μ moles) and (PPh₃)₂PdCl₂ (0.6 mg, 3%) in dry THF (1 ml) under argon at room temperature were treated with acetic acid (3.5 μ l, 2 equiv.) and then with Bu₃SnH (10 μ l, 1.2 equiv.). The reaction was stirred at room temperature for about 1.5 hours and then treated with 1M HCl in ether (60 μ l, 2 equiv.). The mixture was stored in the freezer for about 1 hour and then the crud orange solid was collected by filtration and washed repeatedly with ether. The solid was washed through the glass frit with 5:1 CH₂Cl₂/CH₃OH and then the filtrate was evaporated. The residue was sonicated in methanol (5 ml) to dissolve as much as possible and then filtered to remove an insoluble red

by-product. The filtrate was evaporated to give an orange-red solid (25.1 mg, 75%). ¹H-NMR (CDCl₃/CD₃OD): δ 1.18 (2H, d, sugar CH₃), 1.34, 1.65 and 1.73 (6H, m, Lys CH₂), 2.14 (2H, m, sugar CH₂), 2.81 (2H, m, CH₂-NH₃₊), 3.76 (1H, m, sugar HO-CH), 3.98 (3H, s, OCH₃), 4.05 (1H, m, HN-CH), 4.38 and 4.45 (each 1H, m, Phe and Lys CH), 4.67 (2H, s, CO-CH₂-OH), 4.85 (1H, m, anomeric CH), 7.04 and 7.20 (10H, m, Z and Phe Ph), 7.14 and 7.43 (4H, m, PAB Ph), 7.30, 7.69 and 7.92 (each 1H, m, DOX Ph); HPLC (C-18, 15 cm column, 8:2 MeOH/50 mM Et₃N-HCO₂H buffer (pH 2.8), 1 ml/min., 495 nm): single peak, retention time 7.1-7.2 min.; MS (FAB): 1102 (MH⁺), 1124 (M + Na)⁺; HRMS (FAB): Accurate mass calc. for C₅₈H₆₄N₅O₁₇: 1102.4297, found: 1102.4260.

10 EXAMPLE 10

Preparation of Z-Val-NHS (10)

Z-Val (699.4 mg, 2.78 mmoles) and NHS (352.4 mg, 1.1 equiv.) in THF (20 ml) at about 0°C were treated with DCC (632 mg, 1.1 equiv.). The reaction was worked up as described above for Z-Phe-NHS (4) to give the product as a glassy solid which was carried on to the next step without purification. ¹H-NMR δ 1.03 (6H, 2 x d, Val CH₃), 2.31 (1H, m, Val CH₃-CH), 2.82 (4H, s, NHS CH₂), 4.65 (1H, AB Q, Val CO-CH), 5.12 (2H, s, Z CH₂), 5.30 (1H, d, NH), 7.34 (5H, m, Ph).

20 EXAMPLE 11

Preparation of Z-Val-N'-alloc-Lys (11)

Z-Val-NHS (10) (about 2.78 mmoles) in DME (30 ml) was added to a solution of N'-alloc-Lys-TFA (3) (958.3 mg, 1 equiv.) and NaHCO₃ (468 mg, 2 equiv.) in water (20 ml). The reaction was worked up as described above for Z-Phe-N'-alloc-Lys (5) to give the product as a white solid (1.2855g, quant.). ¹H-NMR (CDCl₃/CD₃OD): δ 0.89 (6H, 2 x d, Val CH₃), 1.30, 1.42, 1.62 and 1.81 (6H, m, Lys CH₂), 2.03 (1H, m, Val CH₃-CH), 3.07 (2H, m, Lys N-CH₂), 3.92 (1H, AB q, Lys CH), 4.42 (1H, m, Val CO-CH), 4.49 (2H, d, allyl O-CH₂), 5.06 (2H, s, Z CH₂), 5.19 (2H, q, vinyl CH₂), 5.82 (1H, m, vinyl CH), 7.28 (5H, m, Ph); MS (FAB): 949 (MH⁺), 971 (M + Na)⁺, 987 (M + K)⁺.

35

Anal. Calc. for C ₂₃ H ₃₃ N ₃ O ₇ :			
Found:	C-59.60, C-59.94,	H-7.18, H-7.31,	N-9.07; N-8.90.

EXAMPLE 12

40 Preparation of Z-Val-N'-alloc-Lys-PAB-OH (12)

Z-Val-N'-alloc-Lys (11) (587.9 mg, 1.27 mmoles) and p-aminobenzyl alcohol (172 mg, 1.1 equiv.) in THF (20 ml) at room temperature were treated with EEDQ (345 mg, 1.1 equiv.). The mixture was stirred at room temperature for 16 hrs. Workup as described above for Z-Phe-N'-alloc-Lys-PAB-OH (6) gave the product as a white solid (591.0 mg, 82%). ¹H-NMR (CDCl₃/CD₃OD): δ 0.86 (6H, m, Val CH₃), 1.24-1.67 (6H, m, Lys CH₂), 2.03 (m, 1H, Val CH₃-CH), 3.08 (2H, m, Lys N-CH₂), 4.00 (1H, m, Lys CH), 4.47 (3H, m, Val CO-CH and allyl O-CH₂), 4.57 (2H, s, PAB-CH₂-OH), 5.05 (2H, s, Z CH₂), 5.19 (2H, q, vinyl CH₂), 5.81 (1H, m, vinyl CH), 7.26 and 7.43 (4H, m, PAB Ph), 7.30 (5H, s, Z Ph); MS (FAB): 569 (MH⁺), 591 (M + Na)⁺, 607 (M + K)⁺.

55

Anal. Calc. for C ₃₀ H ₄₀ N ₄ O ₇ -1/2 H ₂ O:			
Found:	C-62.38, C-62.40,	H-7.15, H-7.22,	N-9.70; N-9.79.

EXAMPLE 13Preparation of Z-Val-N^t-alloc-Lys-PABC-PNP (13)

5 Z-Val-Ne-alloc-Lys-PAB-OH (12) (297.4 mg, 523 μ moles) and p-nitrophenyl chloroformate (264 mg, 2.5 equiv.) in CH₂Cl₂ (15 ml) at room temperature were treated with pyridine (106 μ l, 2.5 equiv.). The mixture was stirred at room temperature for about 16 hours. Workup as described above for Z-Phe-Ne-alloc-Lys-PABC-PNP (7) gave the product as a white solid (271.0 mg, 71%). ¹H-NMR (CDCl₃/CD₃OD): δ 0.91 (6H, m, Val CH₃), 1.33-1.87 (6H, Lys CH₂), 2.02 (1H, m, Val CH₃-CH), 3.08 (2H, m, Lys N-CH₂), 3.95 (1H, m, Lys CH), 4.41 (1H, m, Val CO-CH), 4.48 (2H, d, allyl O-CH₂), 5.06 (2H, s, Z CH₂), 5.17 (2H, q, vinyl CH₂), 5.20 (2H, s, PAB CH₂), 5.82 (1H, m, vinyl CH), 7.23 and 7.58 (4H, m, PAB Ph), 7.30 (5H, m, Z Ph), 7.38 and 8.31 (4H, m, PNP Ph); MS (FAB): 734 (MH⁺), 756 (M + Na)⁺, 772 (M + K)⁺; Accurate mass calc. for C₃₇H₄₄N₅O₁₁: 734.3037; found: 734.3036.

EXAMPLE 14Preparation of Z-Val-N^t-alloc-Lys-PABC-DOX (14)

20 Z-Val-N^t-alloc-Lys-PABC-PNP (13) (260.0 mg, 354 μ moles) and DOX-HCl (216 mg, 1.05 equiv.) in NMP (12 ml) at room temperature were treated with triethylamine (52 μ l). The mixture was allowed to stand in the dark for 2 days. Workup as described above for Z-Phe-N^t-alloc-Lys-PABC-DOX (8) gave the product as an orange solid (278.0 mg, 69%). ¹H-NMR (CDCl₃/CD₃OD): δ 0.83 (6H, m, Val CH₃), 1.18 (3H, d, sugar CH₃), 1.29, 1.41, 1.63 and 1.79 (6H, m, Lys CH₂), 1.72 (2H, m, D-ring CH₂), 1.98 (1H, m, Val CH₃-CH), 2.14 (2H, D-ring CH₂), 3.03 (2H, q, sugar CH₂), 3.02 (2H, m, Lys N-CH₂), 3.52 (1H, m, sugar HO-CH), 3.76 (1H, m, sugar N-CH), 3.94 (1H, m, Lys CH), 3.99 (3H, s, O-CH₃), 4.39 (1H, m, Val CO-CH), 4.42 (2H, d, allyl O-CH₂), 4.69 (2H, s, PAB CH₂), 4.88 (2H, m, Z CH₂), 5.01 (2H, d CO-CH₂-OH), 5.14 (2H, q, vinyl CH₂), 5.18 (1H, m, anomeric CH), 5.41 (1H, br, DOX Ph-CH), 5.80 (1H, m, vinyl CH), 7.13 and 7.40 (4H, PAB Ph), 7.26 (5H, s, Z Ph), 7.32, 7.70 and 7.93 (each 1H, m, DOX Ph), 9.25 (1H, br, PAB NH); MS (FAB) 1160 (M + Na)⁺, 1176 (M + K)⁺; Accurate mass calc. for C₅₈H₆₇N₅O₁₉: 1160.4328; found: 1160.4358.

EXAMPLE 15Preparation of Z-Val-Lys-PABC-DOX-HCl (15)

35 Z-Val-N^t-alloc-Lys-PABC-DOX (14) (84.3 mg, 74.06 μ moles) in THF (2 ml) under argon at room temperature was treated with Pd(PPh₃)₄ (220 μ l of a solution of Pd₂dba₃ (4.7 mg, 5.13 μ moles) and PPh₃ - (13.5 mg, 10 equiv.) in THF (1 ml) under argon), acetic acid (11 μ l, 2.5 equiv.) and tributyltin hydride (30 μ l, 1.5 equiv.). The mixture was stirred at room temperature in the dark for about 1 hour during which time an orange solid began to form. The mixture was diluted with ether (2 ml) followed by 1M HCl in ether (1 ml) and then more ether (25 ml). The resulting suspension was sonicated briefly and then filtered. The orange solid was washed repeatedly with ether and then dissolved in 5:1 CH₂Cl₂/CH₃OH. To this was added celite (about 2 g) and the solvents were evaporated. The resulting solid was dry-loaded atop a celite column (from a slurry in 100:1 CH₂Cl₂/CH₃OH). The column was eluted with 1) 100:1 and 2) 10:1 CH₂Cl₂/CH₃OH, to give the product as an orange solid (58.5 mg, 72.4%). ¹H-NMR (selected peaks)(CDCl₃/CD₃OD): δ (loss of allyl peaks) 0.83 (6H, m, Val CH₃), 1.20 (3H, d, sugar CH₃), 2.02 (1H, m, Val CH₃-CH), 4.01 (3H, s, O-CH₃), 7.10-7.57 (9H, m, Ph), 7.32, 7.72 and 7.91 (each 1H, m, DOX Ph); HPLC (C-18, 15 cm column, 8:2 MeOH/50 mM Et₃N-HCO₂H buffer (pH 2.8), 1 ml/min., 495 nm): single peak, retention time 6.1-6.4 min.; MS (FAB) 1054 (MH)⁺; Accurate mass calc. for C₅₄H₆₄N₅O₁₇: 1054.4297; found: 1054.4283.

EXAMPLE 16Preparation of Alloc-D-Phe (16)

55 D-Phe (2.0203 g, 12.29 mmoles) and NaHCO₃ (1.08 g, 1.05 equiv.) in water (30 ml) were treated with diallyl dicarbonat (2.13 ml, 1.05 equiv.) in DME (30 ml). The mixture was stirred at room temperature for about 16 hours and then poured into 15% citric acid. The resulting suspension was extracted with EtOAc (2 x 100 ml). The combined organic layers were washed with water (3 x 100 ml) and brine, dried and evaporated to give a colorless foam which was pure enough to carry on to the next step (3.002 g, 98%). ¹H-

NMR ($\text{CDCl}_3/\text{CD}_3\text{OD}$): δ 3.13 (2H, AB q, Phe CH_2), 4.52 (2H, d, $\text{CH}_2\text{-O}$), 4.64 (1H, q, Phe CH), 5.20 (2H, q, vinyl CH_2), 5.85 (1H, m, vinyl CH), 7.21 (5H, m, Ph); MS (DCI): 250 (MH^+), 192 ($\text{M-C}_3\text{H}_5\text{O}^+$);

5

Anal. calc. for $\text{C}_{13}\text{H}_{15}\text{NO}_4\text{-H}_2\text{O}$:			
	C-58.42, Found: C-58.81,	H-6.40, H-5.87,	N-5.24; N-5.36.

10

EXAMPLE 17Preparation of Alloc-D-Phe-NHS (17)

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Alloc-D-Phe (16) (3.002 g, 12.04 mmoles) and NHS (1.525 g, 1.1 equiv.) in CH_2Cl_2 at about 0°C were treated with DCC (2.733 g, 1.1 equiv.). The ice bath was allowed to warm to room temperature and the mixture was stirred at room temperature for about 16 hours. Workup as described above for Z-Phe-NHS (4) gave the product as a colorless foam which was used without further purification (4.045 g, 97%).

20

EXAMPLE 18Preparation of Alloc-D-Phe-Phe (18)

25

Alloc-D-Phe-NHS (17) (1.7654 g, 5.10 mmoles) in DME (30 ml) at room temperature was treated with a solution of Phe (1.263 g, 1.5 equiv.) and NaHCO_3 (642.3 mg, 1.5 equiv.) in water (20 ml). The mixture was stirred at room temperature for about 16 hours. The mixture was then poured into 15% citric acid (100 ml) and the resulting suspension was extracted with EtOAc (2 x 100 ml). The combined organic layers were washed with water (3 x) and brine, and then dried and evaporated to give a colorless glass. To this was added ether (30 ml) and the mixture was sonicated at room temperature for about 15 minutes and then stored in the freezer for about 1 hour. The solid product was collected by filtration and washed with ether (1.6973 g, 84%). $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$): δ 2.83-3.16 (4H, m, Ph- CH_2), 4.45 (2H, d, $\text{CH}_2\text{-O}$), 4.63 and 4.89 (each 1H, m, N-CH), 5.21 (2H, q, vinyl CH_2), 5.81 (1H, m, vinyl CH), 6.93-7.34 (10H, m, Ph); MS (DCI): 397 (MH^+);

35

Anal. calc. for $\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_5$:			
	C-66.65, Found: C-66.42,	H-6.10, H-6.19,	N-7.07; N-7.09.

40

EXAMPLE 19Preparation of Alloc-D-Phe-Phe-NHS (19)

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Alloc-D-Phe-Phe (18) (1.0151 g, 2.60 mmoles) and NHS (324.2 mg, 1.1 equiv.) in CH_2Cl_2 (25 ml) at 0°C were treated with DCC (555 mg, 1.05 equiv.). The ice bath was allowed to warm to room temperature and the mixture was stirred for about 18 hours. The solid DCU was removed by filtration and the solvent was evaporated. The residue was dissolved in EtOAc and the solution was washed with water (2x) and brine, dried and evaporated to give a white solid which was used without further purification (1.2897 g, 100%).

50

EXAMPLE 20Preparation of Alloc-D-Phe-Phe-N'-alloc-Lys (20)

55

Alloc-D-Phe-Phe-NHS (19) (1.2897 g, 2.61 mmoles) in DME (40 ml) was added a solution of N'-alloc-Lys-TFA (945 mg, 1.05 equiv.) and NaHCO_3 (461 mg, 2.1 equiv.) in water (20 ml). The mixture was stirred vigorously at room temperature for about 16 hours. Workup as described above for Alloc-D-Phe-Phe (18)

gave a crude white solid. This was suspended in ether and alternately sonicated and heated at about 40 °C for several minutes. The mixture was then stored at about 4 °C for about 2 hours and filtered to remove the white, solid product, which was washed with cold ether (1.2046 g, 76%). ¹H-NMR (CDCl₃/CD₃OD): δ 1.21-1.94 (6H, 4 x m, Lys CH₂), 2.79 and 2.91 (each 2H, m, Phe CH₂), 3.08 (2H, m, N-CH₂), 4.29 (1H, m, Lys CH), 4.38 and 4.59 (each 1H, m, Phe CH), 4.45 and 4.53 (each 2H, d, allyl O-CH₂), 5.20 (4H, m, vinyl CH₂), 5.85 (2H, m, vinyl CH), 7.06-7.27 (10H, m, Ph); MS (FAB): 609 (MH)⁺, 631 (M + Na)⁺, 647 (M + K)⁺;

Anal. calc. for C ₃₂ H ₄₀ N ₄ O ₈ :			
	C-63.14, Found: C-63.05,	H-6.62, H-6.78,	N-9.20; N-9.25.
10			

EXAMPLE 21

Preparation of Alloc-D-Phe-Phe-N'-alloc-Lys-PAB-OH (21)

Alloc-D-Phe-Phe-N'-alloc-Lys (20) (616.8 mg, 1.013 mmoles) and p-aminobenzyl alcohol (137.3 mg, 1.1 equiv.) in THF (12 ml) at room temperature were treated with EEDQ (276 mg, 1.1 equiv.). The mixture was stirred at room temperature for about 18 hours. Workup as described above for Z-Phe-Ne-alloc-Lys-PAB-OH (6) gave the product as a white solid (685.7 mg, 95%). ¹H-NMR (CDCl₃/CD₃OD): δ 1.20-1.98 (6H, 4 x m, Lys CH₂), 2.95 (4H, m, Phe CH₂), 3.08 (2H, m, N-CH₂), 4.25 (2H, AB q, allyl O-CH₂), 4.49 (2H, d, allyl O-CH₂), 4.57 (2H, s, PAB CH₂), 5.15 (4H, m, vinyl CH₂), 5.62 and 5.87 (each 1H, m, vinyl CH), 6.96 and 7.54 (each 2H, m, PAB Ph), 7.06-7.31 (10H, m, Ph); MS (FAB): 714 (MH)⁺, 736 (M + Na)⁺, 752 (M + K)⁺; Accurate mass calc. for C₃₉H₄₈N₅O₈: 714.3503; found: 714.3494;

Anal. calc. for C ₃₉ H ₄₇ N ₅ O ₈ -H ₂ O:			
	C-64.01, Found: C-64.39,	H-6.75, H-6.63,	N-9.57; N-9.54.
30			

EXAMPLE 22

Preparation of Alloc-D-Phe-Phe-N'-alloc-Lys-PABC-PNP (22)

Alloc-D-Phe-Phe-N'-alloc-Lys-PAB-OH (21) (330.8 mg, 463.4 μmoles) and p-nitrophenyl chloroformate (140.1 mg, 1.5 equiv.) in CH₂Cl₂ (20 ml) at room temperature were treated with dry pyridine (56.2 μl, 1.5 equiv.). Workup as described above for Z-Phe-Ne-alloc-Lys-PABC-PNP (7) gave the product as a white solid (379.0 mg, 93%). ¹H-NMR (CDCl₃/CD₃OD): δ 1.20-2.00 (6H, 4 x m, Lys CH₂), 2.97 (4H, m, Phe CH₂), 3.10 (2H, m, N-CH₂), 4.21 (2H, AB q, allyl O-CH₂), 4.30, 4.52 and 4.67 (each 1H, m, N-CH), 4.49 (2H, d, allyl O-CH₂), 5.10 (2H, m, vinyl CH₂), 5.22 (2H, s, PAB CH₂), 5.58 and 5.87 (each 1H, m, vinyl CH), 6.93 and 7.66 (each 2H, m, PAB Ph), 7.04-7.25 (10H, m, Ph), 7.32 and 8.04 (each 2H, m, PNP Ph); MS (FAB): 879 (MH)⁺, 901 (M + Na)⁺, 917 (M + K)⁺; Accurate mass calc. for C₄₆H₅₁N₆O₁₂: 879.3565; found: 879.3533.

EXAMPLE 23

Preparation of Alloc-D-Phe-Phe-N'-alloc-Lys-PABC-DOX (23)

Alloc-D-Phe-Phe-Ne-alloc-Lys-PABC-PNP (22) (379.0 mg, 431.2 mmoles) and DOX-HCl (262.6 mg, 1.05 equiv.) in NMP (10 ml) at room temperature were treated with triethylamine (63 ml, 1.05 equiv.). The mixture was stored in the dark at room temperature for 2 days and then diluted with 10% i-propyl alcohol/EtOAc (150 ml). The resulting solution was washed with water (4x) and brine, filtered to remove a small amount of orange solid by-product, and then evaporated to give an orange solid. This was chromatographed on silica, eluting with 1) 30:1 and 2) 15:1 CH₂Cl₂/CH₃OH, to give the product as an orange solid (418.8 mg, 76%). ¹H-NMR (CDCl₃/CD₃OD): δ 1.21 (3H, d, sugar CH₃), 1.28-1.96 (6H, 4 x m, Lys CH₂), 1.76 (2H, m, D-ring CH₂), 2.18 (D-ring CH₂), 2.87 (2H, m, sugar CH₂), 3.05 (2H, m, N-CH₂), 3.55

(1H, s, sugar HO-CH), 3.78 (1H, m, sugar N-CH), 3.99 (3H, s, CH₃-CH), 4.10 (1H, m, sugar CH₃-CH), 4.26 (2H, m, allyl O-CH₂), 4.40 (3H, m, CO-CH), 4.45 (2H, d, allyl O-CH₂), 4.70 (2H, s, CO-CH₂-OH), 4.89 (2H, m, PAB CH₂), 5.16 (4H, m, vinyl CH₂), 5.20 (1H, s, anomeric CH), 5.41 (1H, s, DOX Ph-CH), 5.52 and 5.80 (each 1H, m, vinyl CH), 6.85-7.52 (14H, m, Ph), 7.32, 7.72 and 7.97 (each 1H, m, DOX Ph); MS (FAB⁻): 5 1282.4 (MH)⁻; Accurate mass calc. for C₆₇H₇₄N₆O₂₀Na: 1305.4856; found: 1305.4877.

EXAMPLE 24Preparation of D-Phe-Phe-Lys-PABC-DOX-2HCl (24)

10 Alloc-D-Phe-Phe-N'-alloc-Lys-PABC-DOX (23) (164.0 mg, 127.8 μ moles) in degassed 2:1 CH₂Cl₂/CH₃OH (4 ml) at room temperature under argon was treated with acetic acid (37 μ l, 5 equiv.) and then 460 μ l of a solution of Pd(PPh₃)₄ (Pd₂dba₃ (6.4 mg) and PPh₃ (18 mg) in degassed 2:1 CH₂Cl₂/CH₃OH (1 ml)). To this was added triethylsilane (61 μ l, 3 equiv.) and the mixture was stirred in the 15 dark for about 16 hours at room temperature. The solvents were removed on the rotovap (40 °C) and the orange, glassy residue was treated with ether (2 ml) and 1M HCl in ether (1 ml). This was sonicated for several minutes. The resulting orange solid was collected by filtration and then taken up as far as possible in water. The insoluble material was filtered off and the filtrate evaporated to dryness. The residue was chromatographed on celite, eluting with 1) 50:1, 2) 12:1, and 3) 5:1 CH₂Cl₂/CH₃OH. The first solvent system 20 eluted any uncharged material, the second eluted singly-charged (mono-deprotected) material, and the product eluted in the third (100.4 mg, 66%). ¹H-NMR (CDCl₃/CD₃OD): δ 1.12 (3H, d, sugar CH₃), 1.00-1.90 (8H, m, Lys CH₂ and D-ring CH₂), 2.07 (2H, m, D-ring CH₂), 2.55-3.16 (8H, m, ¹⁴N-CH₂ sugar CH₂, Phe CH₂), 3.45 (1H, s, sugar HO-CH), 3.70 (1H, m, sugar N-CH), 3.90 (3H, s, O-CH₃), 4.21, 4.33 and 4.43 (each 1H, m, CO-CH), 4.61 (2H, s, CO-CH₂-OH), 4.80 (2H, m, PAB CH₂), 5.12 (1H, brs, anomeric CH), 5.33 (1H, brs, DOX Ph-CH), 6.80-7.90 (17H, m, Ph); HPLC: (C-18, 15 cm column, 8:2 MeOH/50 mM Et₃N-HCO₂H buffer (pH 2.8), 1 ml/min., 495 nm): single peak, retention time 5.5-5.8 min.; MS (FAB⁻): 1114.6 (MH)⁻.

25

EXAMPLE 25Preparation of Z-Val-Cit (26)

To a solution of Z-Val-NHS (10) (2.98 g, 8.566 mmoles) in DME (25 ml) at room temperature was added a solution of citrulline (2.25 g, 1.5 equiv.) and NaHCO₃ (1.08 g, 1.5 equiv.) in water (25 ml). The mixture was stirred vigorously for 2 days. Water (20 ml) containing 2 ml sat. NaHCO₃ was added and the mixture was 35 washed with EtOAc and acidified to pH 3 with 10% HCl. The resulting suspension was extracted with 10% Bu-OH/EtOAc (3x). The combined organic layers were dried and evaporated to give a white solid (3.39 g, 97%). ¹H-NMR (CDCl₃/CD₃OD): δ 0.73 (6H, q, Val CH₃), 1.31, 1.46 and 1.63 (4H, m, Cit CH₂), 1.87 (1H, m, Val CH₃-CH), 2.88 (2H, m, N-CH₂), 3.72 (1H, AB q, Cit CH), 4.17 (1H, m, Val COCH), 4.86 (2H, s, Z CH₂), 40 7.10 (5H, m, Z Ph); MS (FAB): 409 (MH)⁺, 431 (M+Na)⁺, 447 (M+K)⁺; Accurate mass calc. for C₁₉H₂₉N₄O₆: 409.2087; found: 409.2086.

EXAMPLE 26Preparation of Z-Val-Cit-PAB-OH (27)

45 Z-Val-Cit (26) (1,0397 g, 2.545 mmoles) and p-aminobenzyl alcohol (470.2 mg, 1.5 equiv.) in THF (10 ml) at room temperature were treated with EEDQ (944.2 mg, 1.5 equiv.). The mixture was stirred at room temperature for about 16 hours and then diluted with 10% i-Pr-OH/EtOAc (100 ml). This was washed with 50 10% citric acid, water and brine, dried and evaporated. The pale-yellow solid residue was sonicated in ether for 15 min. and the crude solid product was collected by filtration (954.2 mg, 73%). ¹H-NMR (CDCl₃/CD₃OD): δ 0.79 (6H, q, Val CH₃), 1.37, 1.53 and 1.72 (4H, m, Cit CH₂), 1.92 (1H, m, Val CH₃-CH), 3.00 (2H, m, N-CH₂), 3.85 (1H, m, Cit CH), 4.41 (1H, m, Val COCH), 4.45 (2H, s, PAB CH₂), 4.95 (2H, m, Z CH₂), 7.08-7.40 (9H, m, Ph); MS (FAB): 514 (MH)⁺, 536 (M+Na)⁺, 552 (M+K)⁺;

55

5

Anal. calc. for C ₂₆ H ₃₅ N ₅ O ₆ -H ₂ O:			
Found:	C-58.74, C-59.01,	H-7.01, H-6.62,	N-13.17; N-13.17.

EXAMPLE 27Preparation of Z-Val-Cit-PABC-PNP (28)

Z-Val-Cit-PAB-OH (27) (383.0 mg, 745.7 μ moles) and p-nitrophenyl chloroformate (225.5 mg, 1.5 equiv.) in THF (10 ml) and CH₂Cl₂ (5 ml) were treated at room temperature with pyridine (91 μ l, 1.5 equiv.). Workup as described above for Z-Phe-Ne-alloc-Lys-PABC-PNP (7) gave a crude, pale-yellow solid which was chromatographed on silica, eluting with 1) 30:1 and 2) 12:1 CH₂Cl₂/CH₃OH, to give the product as an off-white solid (440.3 mg, 87%). ¹H-NMR (CDCl₃/CD₃OD): δ 0.88 (6H, m, Val CH₃), 1.42, 1.61 and 1.80 (4H, m, Cit CH₂), 2.02 (1H, m, Val CH₃-CH), 3.08 (2H, m, N-CH₂), 3.99 (1H, m, Cit CH), 4.51 (1H, m, Val COCH), 5.00 (2H, m, Z CH₂), 7.20-7.57 (9H, m, Ph), 7.30 and 8.20 (each 2H, m, PNP Ph); MS (FAB): 679 (MH)⁺, 701 (M + Na)⁺, 717 (M + K)⁺; Accurate mass calc. for C₃₃H₃₉N₅O₁₀: 679.2728; found: 679.2720.

EXAMPLE 28Preparation of Z-Val-Cit-PABC-DOX (29)

Z-Val-Cit-PABC-PNP (28) (126.9 mg, 187 μ moles) and DOX-HCl (119.3 mg, 1.1 equiv.) in NMP (5 ml) at room temperature were treated with triethylamine (29 μ l, 1.1 equiv.). The mixture was stirred in the dark at room temperature for 2 days. Workup as described above for Alloc-D-Phe-Phe-N'-alloc-Lys-PABC-DOX (23) gave a crude orange solid. This was chromatographed on silica, eluting with 1) 12:1, 2) 8:1, and 3) 5:1 CH₂Cl₂/CH₃OH, to give the product as a red-orange solid (158.0 mg, 78%). ¹H-NMR (CDCl₃/CD₃OD): δ 0.74 (6H, m, Val CH₃), 1.07 (3H, d, sugar CH₃), 1.28-1.88 (4H, m, Cit CH₂), 1.64 and 2.08 (each 2H, m, D-ring CH₂), 1.88 (1H, m, Val CH₃-CH), 2.87 (2H, m, sugar CH₂), 3.42 (1H, brs, sugar HO-CH), 3.95 (1H, m, sugar N-CH), 4.11 (3H, s, O-CH₃), 4.38 (2H, m, CO-CH), 4.58 (2H, s, CO-CH₂-OH), 4.78 (2H, s, PAB CH₂), 4.90 (2H, s, Z CH₂), 5.04 (1H, brs, anomeric CH), 5.30 (1H, brs, DOX Ph-CH), 7.00-7.86 (12H, m, Ph), 9.31 (1H, brs, PAB NH); NPLC: (C-18, 15 cm column, 8:2 MeOH/50 mM Et₃N-HCO₂H buffer (pH 2.8), 1 ml/min., 495 nm): single peak, retention time 3.65-3.75 min.; MS, (FAB⁻): 1082.8 (M⁻); Accurate mass calc. for C₅₄H₆₃N₆O₁₈: 1083.4199; found: 1083.4161.

EXAMPLE 29Preparation of Z-Phe-N'-alloc-Lys-PABC-2'-Taxol (30)

Taxol (15.8 mg, 18.5 μ moles) and Z-Phe-N'-alloc-Lys-PABC-PNP (7) (14.5 mg, 1 equiv.) in CH₂Cl₂ (2 ml) at room temperature were treated with DMAP (2.5 mg, 1.1 equiv.). After 2 days at room temperature TLC (silica; 25:1 CH₂Cl₂/CH₃OH) indicated completion. EtOAc (25 ml) was added and the mixture was washed with 10% citric acid, water, brine, dried and evaporated to give a pale-yellow glass. This was chromatographed on silica, eluting with 30:1 CH₂Cl₂/CH₃OH, to give the product as a colorless glass (26.1 mg, 94%). ¹H-NMR (selected peaks): δ 1.13, 1.23, 1.68 and 1.81 (each 3H, s, Taxol CH₃), 2.20 and 2.46 (each 3H, s, Ac CH₃), 3.13 (2H, m, CON-CH₂), 4.25 (2H, AB q, C-20 CH₂), 4.47 (1H, m, C-7 CH), 4.52 (2H, d, alloc O-CH₂), 4.97 (2H, m, Z CH₂), 5.05 (2H, s, PAB CH₂), 5.12 (2H, m, vinyl CH₂), 5.45 (1H, d, C-2' CH), 5.88 (1H, m, vinyl CH), 7.10-8.17 (29H, m, Ph), 8.59 (1H, s, PABC NH); MS (ion spray): 1496.8 (MH)⁺, 1519.6 (M + Na)⁺; Accurate mass calc. for C₈₂H₈₉N₅O₂₂: 1496.6078; found: 1496.6082.

EXAMPLE 30Preparation of Z-Phe-Lys-PABC-2'-Taxol-HCl (31)

Z-Phe-N'-alloc-Lys-PABC-2'-Taxol (30) (18.1 mg, 12.09 μ moles) in dry THF (1 ml) at room temp rature under argon was treated with AcOH (1.7 μ l, 2.5 equiv.), Pd(PPh₃)₄ (45 μ l of a solution of Pd₂dba₃ (6.2 mg, 6.77 μ mol s) and PPh₃ (17.8 mg, 10 equiv.) in dry THF (1 ml)), and Bu₃SnH (5 μ l, 1.5 equiv.). After about

30 minutes more Bu_3SnH (5 μ l) was added. After about 30 more minutes ether (5 ml) and then 1M HCl in ether (1 ml) were added. The resulting suspension was sonicated for several minutes and the white solid was collected by filtration and washed repeatedly with ether (14.37 mg, 82%). 1H -NMR ($CDCl_3/CD_3OD$): (selected peaks): δ (loss of allyl peaks) 2.98 (2H, m, $^1H_3N-CH_2$), 4.27 (2H, AB q, C-20 CH₂), 4.39 (1H, m, C-7 CH), 5.02 (2H, m, Z CH₂), 5.09 (2H, m, PAB CH₂), 7.06-8.20 (29H, m, Ph); HPLC: (C-18, 15 cm column, 8:2 MeOH/50 mM Et₃N-HCO₂H buffer (pH 2.8), 1 ml/min., 230 nm): single peak, retention time 4.8 min., (6:4 MeCN/50 mM Et₃N-HCO₂H buffer (pH 2.8)): single peak, retention time: 9.6 min.; MS (ion spray): 1413.2 (MH)⁺; Accurate mass calc. for $C_{78}H_{86}N_5O_{20}$: 1412.5866; found: 1412.5883.

10 EXAMPLE 31

Preparation of Boc-Phe-NHS (32)

15 Boc-Phe (5.4257 g, 20.45 mmoles) and NHS (2.354 g, 1 equiv.) in THF (55 ml) at about 0 °C were treated with DCC (4.22 g, 1 equiv.). The ice bath was allowed to melt and the mixture was stirred at room temperature for about 16 hours. The solid DCU was filtered off and the filtrate was evaporated to give a white solid which was used without further purification (7.2624 g, 98%). 1H -NMR: δ 1.39 (9H, s, t-Bu), 2.85 (4H, br s, NHS CH₂), 3.22 (2H, m, Phe CH₂), 4.94 (1H, m, CH), 7.29 (5H, m, Ph).

20 EXAMPLE 32

Preparation of Boc-Phe-N^t-Fmoc-Lys (33)

25 N^t-Fmoc-Lys (3.0651 g, 8.32 mmoles) and NaHCO₃ (769 mg, 1.1 equiv.) in water (50 ml) and DME (40 ml) were treated, at room temperature, with a solution of Boc-Phe-NHS (32) (3.015 g, 1 equiv.) in DME (40 ml). The mixture was stirred vigorously at room temperature for about 18 hours and then diluted with EtOAc (100 ml) and 10% citric acid. The aqueous layer was re-extracted with EtOAc (50 ml). The combined organic layers were washed with water (2x) and brine, dried and evaporated to give a pale-yellow solid. This was dissolved in ether and a small amount of undissolved solid was removed by filtration. The filtrate was 30 evaporated to dryness and the pale-yellow foamy residue was dried in vacuo (5.0881 g, 99%). 1H -NMR ($CDCl_3/CD_3OD$): δ 1.30, 1.48, 1.67 and 1.85 (6H, m, Lys CH₂), 1.35 (9H, s, t-Bu), 3.01 (2H, m, Phe CH₂), 3.12 (2H, m, N-CH₂), 4.18 (1H, t, Fmoc CH), 4.36 (2H, d, Fmoc CH₂), 4.41 and 4.50 (each 1H, m, CO-CH), 7.12-7.77 (13H, m, Ph); MS (FAB): 616 (MH)⁺, 638 (M + Na)⁺, 654 (M + K)⁺;

35

Anal. calc. for $C_{35}H_{41}N_3O_7$:			
Found:	C-68.27, C-68.13,	H-6.71, H-6.84,	N-6.82; N-6.44.

40

EXAMPLE 33

Preparation of Boc-Phe-N^t-Fmoc-Lys-PAB-OH (34)

45 Boc-Phe-N^t-Fmoc-Lys (33) (4.8207 g, 7.83 mmoles) and p-aminobenzyl alcohol (1.061 g, 1.1 equiv.) in THF (50 ml) at room temperature were treated with EEDQ (2.13 g, 1.1 equiv.). The mixture was stirred at room temperature for about 16 hours. Workup as described above for Z-Phe-N^t-alloc-Lys-PAB-OH (6) gave the product as an off-white solid (4.4579 g, 79%). 1H -NMR ($CDCl_3/CD_3OD$): δ 1.28, 1.48, 1.63 and 1.84 (6H, m, Lys CH₂), 1.33 (9H, s, t-Bu), 3.00 (2H, m, Phe CH₂), 3.11 (2H, m, N-CH₂), 4.15 (1H, t, Fmoc CH), 4.31 (2H, d, Fmoc CH₂), 4.38 (2H, m, CO-CH), 4.57 (2H, s, PAB CH₂), 7.08-7.75 (17H, m, Ph); MS (FAB): 721 (MH)⁺, 743 (M + Na)⁺, 759 (M + K)⁺;

55

Anal. calc. for $C_{42}H_{48}N_4O_7 \cdot 1/2 H_2O$:			
Found:	C-69.12, C-68.96,	H-6.77, H-6.87,	N-7.68; N-7.64.

EXAMPLE 34Preparation of 2'-Fmoc-Taxol (35)

5 Taxol (134.6 mg, 157.6 μ moles) and Fmoc-NHS (58.5 mg, 1.1 equiv.) in CH_2Cl_2 (3 ml) at room temperature were treated with DMAP (19.3 mg, 1 equiv.). After about 5 days at room temperature TLC (silica; 25:1 $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$) indicated completion. EtOAc (50 ml) was added and the mixture was washed with 10% citric acid, water, brine, dried and evaporated. The residue was chromatographed on silica, eluting with 35:1 $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$, to give the product as a colorless glass (165.6 mg, 98%). $^1\text{H-NMR}$: δ 1.13, 1.24 and 1.67 (each 3H, s, C-16, C-17 and C-19 CH_3), 1.92 (3H, s, C-18 CH_3), 1.87 and 2.52 (2H, m, C-6 CH_2), 2.22 and 2.44 (each 3H, s, Ac CH_3), 2.41 (2H, m, C-14 CH_2), 2.50 (1H, d, C-7 OH), 3.82 (1H, d, C-3 CH), 4.28-4.51 (6H, m, C-20 CH_2 , C-7 CH, Fmoc CH and CH_2), 4.98 (1H, d, C-5 CH), 5.47 (1H, d, C-2' CH), 5.69 (1H, d, C-2 CH), 6.03 (1H, m, C-3' CH), 6.30 (1H, s, C-10 CH), 6.32 (1H, t, C-13 CH), 6.99 (1H, d, NH), 7.22-8.20 (23H, m, Ph); MS (FAB): 1076 (MH^+), 1098 ($\text{M} + \text{Na}^+$), 1114 ($\text{M} + \text{K}^+$); Accurate mass calc. for $\text{C}_{26}\text{H}_{62}\text{NO}_{16}$: 1076.4069; found: 1076.4031.

EXAMPLE 35Preparation of Boc-Phe-N'-Fmoc-Lys-PABC-7-Taxol-2'-Fmoc (36)

20 2'-Fmoc-taxol (35) (112.1 mg, 90.3 μ moles) in dry CH_2Cl_2 (1 ml) under argon at about 0 °C was treated with pyridine (8 μ l, 1.1 equiv.) and diphosgene (6.5 μ l, 0.6 equiv.). After about 40 minutes Boc-Phe-N'-Fmoc-Lys-PAB-OH (65.1 mg, 1 equiv.) and DMAP (0.5 mg) in CH_2Cl_2 (1 ml)/pyridine (0.2 ml) were added. The mixture was stirred at about 0 °C for about 30 minutes and then at room temperature for about 4 hours. 25 EtOAc (30 ml) was then added and the solution was washed with 10% citric acid (2 x), water and brine, then dried and evaporated to give a white solid. This was chromatographed on silica, eluting with 30:1 $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$, to give the product as a colorless glass (81.7 mg, 50%, two of the three product-containing fractions were contaminated with 2'-Fmoc-taxol). $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$): δ 1.19, 1.22 and 1.80 (each 3H, s, C-16, C-17 and C-19 CH_3), 1.10-1.90 (6H, m, Lys CH_2), 1.38 (9H, s, t-Bu), 1.82 and 2.54 (each 1H, m, C-6 CH_2), 2.05 (3H, s, C-18 CH_3), 2.23 and 2.42 (each 1H, m, C-14 CH_2), 2.18 and 2.47 (each 3H, s, Ac CH_3), 3.09 (2H, m, Phe CH_2), 3.19 (2H, m, Lys N- CH_2), 3.98 (1H, d, C-3 CH), 4.15-4.52 (7H, m, Phe and Lys CO-CH, Fmoc CH_2 and CH, C-20 CH_2), 4.98 (1H, m, C-5 CH), 5.14 (2H, m, PAB CH_2), 5.48 (1H, d, C-2' CH), 5.55 (1H, m, C-7 CH), 5.69 (1H, m, C-2 CH), 6.02 (1H, m, C-3' CH), 6.29 (1H, m, C-13 CH), 6.41 (1H, s, C-10 CH), 6.96-8.18 (40H, m, Ph); MS (FAB): 1823 (MH^+), 1846 ($\text{M} + \text{Na}^+$), 1862 ($\text{M} + \text{K}^+$).

EXAMPLE 36Preparation of Boc-Phe-Lys-PABC-7-Taxol-HCl (37)

40 Boc-Phe-N'-Fmoc-Lys-PABC-7-Taxol-2'-Fmoc (36) (74.6 mg, 40.95 μ moles) in THF (2 ml) at room temperature was treated with 2% DBU in THF (2 ml). After about 6 minutes at room temperature ether (25 ml) was added and the resulting white solid was collected by filtration and washed with ether. The solid was suspended in ether (5 ml) and treated with 1M HCl in ether (2 ml). After about 2 minutes the solid was filtered off and washed thoroughly with ether. The solid was chromatographed on LH-20 lipophilic sephadex, 45 eluting with 1:1 $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$, to give the product as a colorless glass (35.6 mg, 90%). $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$): δ 1.13, 1.19 and 1.78 (each 3H, s, C-16, C-17 and C-19 CH_3), 1.37 (9H, s, t-Bu), 1.10-1.90 (6H, m, Lys CH_2), 1.86 and 2.54 (each 1H, m, C-6 CH_2), 2.05 (3H, s, C-18 CH_3), 2.16 and 2.38 (each 3H, s, Ac CH_3), 2.97 (2H, m, $^+{\text{H}_3\text{N}-\text{CH}_2}$), 3.12 (2H, m, Phe CH_2), 3.90 (1H, d, C-3 CH), 4.24 (2H, m, C-20 CH_2), 4.45 and 4.68 (each 1H, m, Phe and Lys CO-CH), 4.83 (1H, brs, C-2' CH), 4.91 (1H, d, C-5 CH), 5.12 (2H, m, PAB CH_2), 5.48 (1H, m, C-7 CH), 5.67 (1H, d, C-2 CH), 5.78 (1H, d, C-3' CH), 6.12 (1H, m, C-13 CH), 6.33 (1H, s, C-10 CH), 7.08-8.12 (24H, m, Ph); HPLC: (C-18, 15 cm column, 8:2 MeOH/50 mM $\text{Et}_3\text{N}-\text{HCO}_2\text{H}$ buffer (pH 2.8), 1 ml/min., 230 nm): single peak, retention time: 7.1-7.3 min.; MS (ion spray): 1379.2 (MH^+); Accurate mass calc. for $\text{C}_{75}\text{H}_{88}\text{N}_5\text{O}_{20}$: 1378.6023; found: 1378.6043.

EXAMPLE 37Preparation of Boc-Phe-N^o-Fmoc-Lys-PABC-Cl (38)

5 Boc-Phe-N^o-Fmoc-Lys-PAB-OH (34) (211.2 mg, 293 μ moles) in pyridine (0.5 ml) and CH₂Cl₂ (2 ml) at -42°C (dry ice-MeCN) under argon was treated with diphosgene (21.2 μ l, 0.6 equiv.). The mixture was stirred at about -42°C for about 20 minutes during which time solid pyridinium hydrochloride had precipitated out of solution. This solution was used immediately.

10 EXAMPLE 38Preparation of Boc-Phe-N^o-Fmoc-Lys-PABC-MMC (39)

15 To the above solution of Boc-Phe-N^o-Fmoc-Lys-PABC-Cl (38) at about -42°C was added MMC (118.0 mg, 1.2 equiv.) in NMP (1 ml). The cooling bath was allowed to warm to room temperature gradually and the mixture was stirred in the dark for about 12 hours at room temperature. The mixture was diluted with 10% i-Pr-OH/EtOAc (50 ml) and 10% citric acid (50 ml). The organic layer was washed with water (3x) and brine, dried and evaporated to give a purple-brown residue. This was chromatographed on a 1 mm silica prep. plate, eluting with 12:1 CH₂Cl₂/CH₃OH, to give the product as a light purple solid (108.0 mg, 34%).
 20 ¹H-NMR (CDCl₃/CD₃OD): δ 1.21, 1.43, 1.61 and 1.81 (6H, m, Lys CH₂), 1.32 (9H, s, t-Bu), 2.10 (3H, s, MMC CH₃), 2.99 (2H, m, Phe CH₂), 3.11 (2H, m, Lys N-CH₂), 3.14 (3H, s, O-CH₃), 3.20-3.50 (3H, m, C-1 and C-2 CH, and C-3 CH), 3.62 (1H, ABq, C-9 CH), 4.18 (1H, t, Fmoc CH), 4.22 and 4.89 (each 1H, ABq, C-10 CH₂), 4.32 (2H, d, Fmoc CH₂), 4.41 (1H, d, C-3 CH), 4.45 (2H, m, Phe and Lys CO-CH), 5.01 (2H, m, PAB CH₂), 7.05-7.90 (17H, m, Ph); MS (FAB): 1082 (MH)⁺, 1103 (M + Na)⁺, 1119 (M + K)⁺; Accurate mass calc. for C₅₈H₆₄N₈O₁₃Na: 1103.4491; found: 1103.4451.

EXAMPLE 39Preparation of Boc-Phe-Lys-PABC-MMC-HCl (40)

30 Boc-Phe-N^o-Fmoc-Lys-PABC-MMC (39) (11.2 mg, 10.36 μ moles) in THF (1 ml) at room temperature was treated with 2% DBU in THF (1 ml). A fine purple solid slowly formed. After about 5 minutes the volume was reduced to about 1 ml on the rotovap (bath temp. 30°C) and ether (10 ml) was added. The resulting solid was collected by filtration and washed with ether. The solid was suspended in ether (2 ml) and treated with 1M HCl in ether (3 ml). After about 2 minutes the solid was filtered off, washed thoroughly with ether, and then triturated with CH₂Cl₂ (2 ml). The resulting solid was collected by filtration and washed with CH₂Cl₂ (9.1 mg, 98%). ¹H-NMR (CDCl₃/CD₃OD): δ 1.30 (9H, s, t-Bu), 1.20-1.90 (6H, m, Lys CH₂), 1.94 (3H, s, MMC CH₃), 2.83 (2H, m, ¹H₃N-CH₂), 2.98 (2H, m, Phe CH₂), 3.13 (3H, s, O-CH₃), 3.20-3.70 (4H, m, C-1 and C-2 CH, C-3 CH and ABq, C-9 CH), 4.14 and 4.82 (each 1H, ABq, C-10 CH), 4.25-4.52 (3H, m, Phe and Lys CO-CH and C-3 CH), 4.97 (2H, m, PAB CH₂), 7.12 (5H, brs, Phe Ph), 7.23 and 7.50 (each 2H, m, PAB Ph); HPLC: (C-18, 15 cm column, 65:35 MeOH/50 mM Et₃N-HCO₂H buffer (pH 2.8), 1 ml/min., 365 nm): single peak, retention time: 4.1-4.3 min.; MS (FAB): 859 (MH)⁺, 881 (M + Na)⁺, 897 (M + K)⁺; Accurate mass calc. for C₄₃H₅₅N₈O₁₁: 859.3990; found: 859.3980.

45 EXAMPLE 40Preparation of N^o-Fmoc-N^o-Mtr-Lys (41)

50 N^o-Fmoc-Lys (14.840 g, 40.28 mmoles) was suspended in dry CH₂Cl₂ (200 ml) at room temperature under argon. Trimethylsilyl chloride (10.9 ml, 2 equiv.) was added with vigorous stirring, and the mixture was heated at reflux for about one hour, and then cooled to about 0°C. DIEA (14.0 ml, 2 equiv.) was added, followed by p-anisylidiphenylmethyl chloride (13.061 g, 1.05 equiv.) in CH₂Cl₂ (50 ml). The ice bath was removed and the mixture was stirred for about 2 hours at room temperature. Methanol (8.2 ml, 5 equiv.) was added and stirring was continued for one hour and then the solvents were evaporated. The residue was partitioned between ethyl acetate and pH 5 buffer (biphenylate). The organic layer was washed with water and brine, dried and evaporated, giving a pale orange gum. This was flushed with CH₂Cl₂ and dried in vacuo to give a foam which was carried on without further purification (25.693 g, 99%). ¹H-NMR (CDCl₃) δ 1.26 and 1.68 (2H and 4H, m, Lys CH₂), 2.45 (2H, m, N-CH₂), 3.71 (3H, s, OCH₃), 4.05-4.40 (4H, m, Fmoc

CH₂ and CH, 6.81 (2H, d, MeOPh o-CH), 7.15-7.77 (2H, m, Ph); MS (FAB) 641 (MH)⁺, 663 (M + Na)⁺, 679 (M + K)⁺.

EXAMPLE 41

5 Preparation of N^o-Mtr-Lys (42)

N^o-Fmoc-N^o-Mtr-Lys (41) (10.006 g, 15.615 mmoles) in CH₂Cl₂ (50 ml) at room temperature was treated with diethylamine (40 ml). The mixture was stirred at room temperature for about 24 hours and then the solvents were evaporated and the residue flushed with CH₂Cl₂ (3x100 ml). The pale yellow residue was triturated with ether. The resulting suspension was sonicated for several minutes, and the solid was collected by filtration, washed with ether and dried in vacuo for several hours (6.191 g, 95%). ¹H-NMR (CDCl₃/CD₃OD) δ 1.34, 1.57 and 1.72 (6H, m, Lys CH₂), 2.05 (2H, m, N-CH₂), 3.38 (1H, m, CO-CH), 3.68 (3H, s, O-CH₃), 3.71 (2H, d, MeOPh o-CH), 7.03-7.40 (12H, m, Ph); MS (FAB) 419.2 (MH)⁺, 441.4 (M + Na)⁺, 457.4 (M + K)⁺.

EXAMPLE 42

20 Preparation of Fmoc-Phe-NHS (43)

Fmoc-Phe (5.1043 g, 13.17 mmoles) and NHS (1.592 g, 1.05 equiv.) in CH₂Cl₂ (100 ml) at about 0°C were treated with DCC (2.854 g, 1.05 equiv.). The ice bath was allowed to warm to room temperature and the mixture was stirred for about 14 hours. The DCU by-product was removed by filtration and the filtrate was evaporated. The resulting crude product, a colorless glass, was used without further purification.

25 EXAMPLE 43

Preparation of Fmoc-Phe-N^o-Mtr-Lys (44)

30 A suspension of N^o-Mtr-Lys (42) (4.686 g, 11.20 mmoles) and NaHCO₃ (941.0 mg, 1 equiv.) in water (100 ml) and DME (50 ml) was treated with a solution of Fmoc-Phe-NHS (43) (11.20 mmoles) in DME (50 ml). THF (25 ml) was then added to aid solubility. The mixture was stirred at room temperature for 2 days and then as much DME as possible was removed on the rotovap (bath at about 30°C). The resulting gummy suspension was partitioned between ethyl acetate and pH 5 buffer. The organic phase was washed 35 with water and brine, dried and evaporated to give a pale yellow foam. This was flushed with CH₂Cl₂ (100 ml). TLC showed the product to be fairly pure and it was carried on without further purification (8.559 g, 97%). ¹H-NMR (CDCl₃/CD₃OD) δ 1.10-1.93 (6H, m, Lys CH₂), 2.31 (2H, t, N-CH₂), 3.00 (2H, m, Phe CH₂), 3.71 (3H, s, O-CH₃), 4.02-4.48 (5H, m, Fmoc CH₂ and CH, CO-CH), 6.79 (2H, d, MeOPh o-CH), 7.00-7.75 (25H, m, Ph); MS (FAB) 788.2 (MH)⁺, 810.4 (M + Na)⁺, 826 (M + K)⁺;

40

Anal. calc. for C ₅₀ H ₄₉ N ₃ O ₆ -H ₂ O:			
Found:	C-74.51, C-74.17,	H-6.38, H-6.57,	N-5.21; N-5.41.

45

EXAMPLE 44

50 Preparation of Fmoc-Phe-N^o-Mtr-Lys-PAB-OH (45)

Fmoc-Phe-N^o-Mtr-Lys (44) (7.728 g, 9.808 mmoles) and p-aminobenzyl alcohol (1.450 g, 1.2 equiv.) in CH₂Cl₂ (100 ml) at room temperature were treated with EEDQ (3.640 g, 1.5 equiv.). The mixture was stirred at room temperature for about 20 hours and then the solvent was evaporated (water bath at about 30°C). The solid residue was triturated with ether (200 ml) and the resulting suspension sonicated for about 15 minutes and left to stand at room temperature for about 2 hours. The resulting solid was collected by filtration, washed well with ether, and dried in vacuo (7.6140 g, 87%). ¹H-NMR (CDCl₃/CD₃OD) δ 0.98-1.91 (6H, m, Lys CH₂), 2.06 (2H, t, N-CH₂), 2.97 (2H, m, Phe CH₂), 3.71 (3H, s, O-CH₃), 4.12 (1H, t, Fmoc-CH), 4.20-4.41 (4H, m, Fmoc CH₂ and CO-CH), 4.59 (2H, s, PAB CH₂), 6.72 (2H, d, MeOPh o-CH), 7.00-7.73

(29H, m, Ph); MS (FAB) 891.4 (MH^+), 916.7 ($M + Na^+$), 931 ($M + K^+$);

Anal. calc. for $C_{57}H_{56}N_4O_6 \cdot H_2O$:			
	C-75.14, C-75.25,	H-6.42, H-6.02,	N-6.15; N-6.49.
5	Found:		

EXAMPLE 45Preparation of Phe-N'-Mtr-Lys-PAB-OH (46)

Fmoc-Phe-N'-Mr-Lys-PAB-OH (45) (4.2857 g, 4.80 mmoles) in CH_2Cl_2 (35 ml) at room temperature was treated with diethylamine (50 ml). The mixture was sonicated briefly and stirred at room temperature for 4 hours after which time no starting material was observed by TLC. The solvents were evaporated and the residue was flushed with CH_2Cl_2 and chromatographed on silica, eluting with 1) 2% methanol/ CH_2Cl_2 , 2) 3% methanol/ CH_2Cl_2 , and 3) 4% methanol/ CH_2Cl_2 , to give the product as a colorless foam (2.230 g, 69%). 1H -NMR ($CDCl_3$) δ 1.26-2.00 (6H, m, Lys CH₂), 2.12 (2H, t, N-CH₂), 2.75 and 3.21 (each 1H, ABq, Phe CH₂), 3.68 (1H, ABq, Phe CO-CH), 3.76 (3H, s, O-CH₃), 4.42 (1H, q, Lys CO-CH), 4.66 (2H, brs, PAB CH₂), 6.79 (2H, d, MeOPh o-CH), 7.10-7.42 (21H, m, Ph), 7.81 (1H, d, amide NH), 8.71 (1H, s, PAB NH); MS (FAB) 693.4 ($M + Na^+$), 709 ($M + K^+$);

Anal. calc. for $C_{42}H_{46}N_4O_4 \cdot 1/2H_2O$:			
	C-74.20, C-74.28,	H-6.97, H-7.00,	N-8.24; N-8.34.
25	Found:		

EXAMPLE 46Preparation of MC-Phe-N'-Mtr-Lys-PAB-OH (47)

Phe-N'-Mtr-Lys-PAB-OH (46) (448.1 mg, 0.668 mmoles) and DIEA (0.128 ml, 1.1 equiv.) in CH_2Cl_2 (5 ml) at room temperature were treated with MC-NHS (230.4 mg, 1.12 equiv.) in CH_2Cl_2 (2 ml). The mixture was stirred at room temperature for 3 days. Ethyl acetate (60 ml) was added and the mixture was washed with pH 5 buffer (2x), water and brine, dried and evaporated. The residue was triturated with ether (60 ml) and the resulting solid collected by filtration and washed with ether (563.8 mg, 98%). 1H -NMR ($CDCl_3$) δ 1.05-1.96 (12H, m, Lys and caproyl CH₂), 2.07 (2H, t, Lys N-CH₂), 2.18 (2H, t, CO-CH₂), 3.02 (2H, m, Phe CH₂), 3.39 (2H, t, M-CH₂), 3.71 (3H, s, O-CH₃), 4.64 (3H, s and m, PAB CH₂ and Lys CO-CH), 4.99 (1H, q, Phe CO-CH), 6.61 (2H, s, M CH), 6.71 (2H, d, MeOPh o-CH), 6.89 (1H, m, amide NH), 7.00-7.55 (21H, m, Ph), 8.97 (1H, brs, PAB NH); MS (FAB) 864 (MH^+), 886 ($M + Na^+$), 902.4 ($M + K^+$).

EXAMPLE 47Preparation of MC-Phe-N'-Mtr-Lys-PABC-PNP (48)

MC-Phe-N'-Mtr-Lys-PAB-OH (47) (679.3 mg, 0.786 mmoles) and bis-p-nitrophenyl carbonate (1.196 g, 5 equiv.) under argon at room temperature were dissolved in CH_2Cl_2 (25 ml) and treated with DIEA (0.411 ml, 3 equiv.). After 3 days TLC indicated completion. The volume was reduced to about 5 ml on the rotovap and the residue was diluted with ethyl acetate (80 ml) and washed with pH 5 buffer, water and brine, dried and evaporated. The resulting solid was triturated with ether (80 ml), and the solid was collected by filtration, washed with ether, and chromatographed on silica, eluting with 1) 1:1 and 2) 8:1 ethyl acetate/hexane (the sample was loaded on the column in a minimum amount of 8:1 ethyl acetate/hexane), to give the product as a pale yellow glass (670.7 mg, 83%). 1H -NMR ($CDCl_3$) δ 1.10-1.95 (12H, m, Lys and caproyl CH₂), 2.04 (2H, t, Lys N-CH₂), 2.13 (2H, t, CO-CH₂), 3.04 (2H, m, Phe CH₂), 3.39 (2H, t, M-CH₂), 3.72 (3H, s, O-CH₃), 4.58 (1H, q, Lys CO-CH), 4.86 (1H, q, Phe CO-CH), 5.27 (2H, s, PAB CH₂), 6.58 (1H, d, amide NH), 6.61 (2H, s, M CH), 6.72 (2H, d, MeOPh o-CH), 7.03-7.62 (27H, m, Ph and NH), 8.22 (2H, d,

PNP CH), 8.86 (1H, brs, PAB NH); MS (FAB) 1029 (MH)⁺, 1051.5 (M + Na)⁺, 1069.4 (M + K)⁺.

EXAMPLE 48

5 Preparation of MC-Phe-N^t-Mtr-Lys-PABC-DOX (49)

MC-Phe-N^t-Mtr-Lys-PABC-PNP (48) (126.6 mg, 0.123 mmoles) and DOX·HCl (71.3 mg, 1 equiv.) in NMP (5 ml) at room temperature were treated with DIEA (21.4 μ L, 1 equiv.). After 2 days standing in the dark at room temperature the mixture was diluted with ethyl acetate (60 ml) and washed with water (4x) and brine, dried and evaporated. The residue was chromatographed on silica, eluting with 1) 25:1 and 2) 20:1 CH_2Cl_2 /methanol, to give the product as an orange glass (149.0 mg, 85%). ¹H-NMR (CDCl_3) δ 1.10-1.95 (14H, m, Lys and caproyl CH_2 , D-ring CH_2), 1.27 (3H, d, sugar CH_3), 2.10 (4H, m, Lys N- CH_2 and caproyl CO- CH_2), 2.23 (2H, m, D-ring CH_2), 3.03 (2H, m, Phe CH_2), 3.20 (2H, m, sugar CH_2), 3.41 (2H, t, M- CH_2), 3.67 (1H, brs, sugar HO-CH), 3.77 (3H, s, Mtr O- CH_3), 4.08 (3H, s, DOX O- CH_3), 4.13 (sugar N-CH), 4.40 (1H, m, Phe CO-CH), 4.56 (2H, m, Lys CO-CH and sugar CH_3 -CH), 4.76 (2H, brs, CO- CH_2 -OH), 4.99 (2H, m, PAB CH_2), 5.29 (1H, brs, anomeric CH), 5.51 (1H, brs, DOX Ph-CH), 5.18, 6.02 and 6.38 (each 1H, m, NH), 6.62 (2H, s, M CH), 6.77 (2H, d, MeOPh o-CH), 7.00-7.60 (22H, m, Ph), 7.78 and 8.03 (each 1H, m, DOX Ph CH), 8.22 (1H, brs, PAB NH); MS (FAB) 1433.8 (MH)⁺, 1456.0 (M + Na)⁺, 1471.8 (M + K)⁺.

20 EXAMPLE 49

Preparation of MC-Phe-Lys-PABC-DOX · Cl₂CHCO₂H (50)

A stirred solution of MC-Phe-N^t-Mtr-Lys-PABC-DOX (49) (1.1520 g, 0.804 mmoles) in CH_2Cl_2 (50 ml) and anisole (8.73 ml, 100 equiv.) was treated with dichloroacetic acid (0.663 ml, 10 equiv.). After about 1 hour ethyl acetate (80 ml) was added and the resulting suspension was stored in the freezer for about 1.5 hours. The solid was collected by filtration, washed with ethyl acetate, and dried in vacuo. The filtrate was concentrated to about 30 ml on the rotovap (bath at about 27°C) and then ether (50 ml) was added. The resulting suspension was stored in the freezer for about 1 hour and then filtered. The orange solid was triturated repeatedly with CH_2Cl_2 and then dried in vacuo (1.0092 g, 97%). ¹H-NMR ($\text{CDCl}_3/\text{CD}_3\text{OD}$) δ 1.10-1.90 (14H, m, Lys and caproyl CH_2 , D-ring CH_2), 1.21 (3H, d, sugar CH_3), 2.10 (2H, t, caproyl CO- CH_2), 2.20 (2H, m, D-ring CH_2), 2.88 (2H, m, Lys N- CH_2), 3.02 (2H, m, Phe CH_2), 3.12 (2H, m, sugar CH_2), 3.38 (2H, t, M- CH_2), 3.52 (1H, brs, sugar HO-CH), 3.79 (1H, m, sugar HN-CH), 4.02 (3H, s, DOX O- CH_3), 4.10 (1H, m, sugar CH_3 -CH), 4.43 and 4.54 (each 1H, m, Phe and Lys CO-CH), 4.72 (2H, s, DOX CO- CH_2 -OH), 4.92 (2H, m, PAB CH_2), 5.24 (1H, br s, anomeric CH), 5.44 (1H, br s, DOX Ph-CH-O-sugar), 5.84 (1H, s, Cl₂CH), 6.67 (2H, s, M CH), 7.10 (5H, brs, Phe Ph), 7.21 and 7.48 (each 2H, d, PAB Ph), 7.38, 7.77 and 7.99 (each 1H, d, t, and d, resp., DOX Ph); HPLC: (C-18, 15 cm column, 8:2 methanol/50 mM triethylammonium formate buffer (pH 2.8), 1 ml/min., 495 nm): single peak, retention time: 4.4-4.5 min.; MS (FAB⁻): 1159 (M-H)⁻; Accurate mass calc. for C₆₀H₆₈N₆O₁₈Na: 1183.4488; found: 1183.4457.

40 EXAMPLE 50

Preparation of MC-Phe-N^t-Mtr-Lys-PABC-MMC (51)

A stirred mixture of MC-Phe-N^t-Mtr-Lys-PABC-PNP (48) (160.4 mg, 0.1559 mmoles), HOEt (211.0 mg, 10 equiv.) and MMC (57.3 mg, 1.1 equiv.) in NMP (5 ml) at room temperature was treated with DIEA (0.271 ml, 10 equiv.). After about 14 hours at room temperature ethyl acetate (100 ml) was added and the mixture was washed with pH 5 buffer, water and brine, dried and evaporated. The residue was chromatographed on silica, eluting with 1) 25:1 and 2) 20:1 CH_2Cl_2 /methanol, to give the product as a purple glass (136.2 mg, 71%). ¹H-NMR (CDCl_3) δ 1.08-1.90 (12H, m, CH_2), 1.73 (3H, s, MMC CH_3), 2.10 (4H, m, Lys N- CH_2 and CO- CH_2), 3.05 (2H, m, Phe CH_2), 3.18 (3H, s, MMC O- CH_3), 3.23-3.50 (5H, m, C-1, C-2 and C-3 CH and M- CH_2), 3.63 (1H, ABq, C-9 CH), 3.74 (3H, s, Mtr O- CH_3), 4.28 and 4.90 (each 1H, t and ABq, C-10 CH_2), 4.41 (2H, d and m, C-3 CH and Phe CO-CH), 4.71 (1H, m, Lys CO-CH), 5.01 (2H, m, PAB CH_2), 5.09 (1H, brs, amide NH), 5.30 (4H, brs, NH₂), 6.31 and 6.88 (each 1H, d, amide NH), 6.63 (2H, s, M CH), 6.76 (2H, d, MeOPh o-CH), 7.06-7.57 (21H, m, Ph), 8.81 (1H, brs, PAB NH); MS (FAB) 1246.5 (M + Na)⁺, 1262.3 (M + K)⁺.

EXAMPLE 51Preparation of MC-Phe-Lys-PABC-MMC • ClCH₂CO₂H (52)

5 A stirred solution of MC-Phe-N'-Mtr-Lys-PABC-MMC (51) (68.1 mg, 55.6 μ moles) in CH₂Cl₂ (3 ml) and anisole (0.604 ml, 100 equiv.) was treated with chloroacetic acid (1M in CH₂Cl₂, 0.56 ml, 10 equiv.). A purple precipitate gradually formed. After 3 hours ether (5 ml) was added. The resulting solid was collected by filtration and washed with ether and CH₂Cl₂, and then dissolved in methanol. HPLC showed it to be >95% pure (44.7 mg, 74%). ¹H-NMR (CDCl₃/CD₃OD) δ 1.11, 1.40, 1.63 and 1.77 (12H, m, CH₂), 2.09 (2H, t, CO-CH₂), 3.02 (2H, m, Phe CH₂), 3.13 (3H, s, MMC O-CH₃), 3.23-3.50 (5H, m, C-1, C-2 and C-3 CH and M-CH₂), 3.56 (1H, ABq, C-9 CH), 3.92 (2H, brs, ClCH₂), 4.13 and 4.82 (each 1H, t and ABq, C-10 CH₂), 4.30 (1H, d, C-3 CH), 4.41 (1H, m, Phe CO-CH), 4.65 (1H, m, Lys CO-CH), 4.99 (2H, q, PAB CH₂), 6.63 (2H, s, M-CH), 7.10 (5H, brs, Phe Ph), 7.22 and 7.48 (each 2H, d, PAB Ph); MS (FAB) 952.3 (MH)⁺, 974 (M + Na)⁺, 990.3 (M + K)⁺; HPLC: (C-18, 15 cm column, 65:35 methanol/50 mM triethylammonium formate buffer (pH 2.8), 1 ml/min., 360 nm): single peak, retention time: 2.84 min.

EXAMPLE 52Preparation of 2'-Methoxytrityl-Taxol (53)

20 A stirred solution of taxol (0.51 g, 0.597 mmoles) and p-methoxytrityl chloride (4.63 g, 25 equiv.) in CH₂Cl₂ (14 ml) under nitrogen at room temperature was treated with pyridine (1.23 ml, 25 equiv.). After about 16 hours at room temperature the solvent was evaporated and the residue dissolved in ethyl acetate. The solution was washed with cold pH 5 buffer (2x100ml), water and brine, dried and evaporated. The residue was chromatographed on silica, eluting with 3% methanol/CH₂Cl₂, to give the product as a white solid (482 mg, 72%). ¹H NMR (CDCl₃) δ 1.11, 1.17 and 1.55 (each 3H, s, C-16, C-17 and C-19 CH₃), 1.67 (3H, s, C-18 CH₃), 1.90 and 2.54 (2H, m, C-6 CH₂), 2.26 and 2.51 (each 3H, s, Ac CH₃), 2.54 (2H, m, C-14 CH₂), 3.66 (1H, d, C-3 CH), 3.78 (3H, s, O-CH₃), 4.21 (2H, ABq, C-20 CH₂), 4.41 (1H, m, C-7 CH), 4.63 (1H, d, C-2' CH), 4.92 (1H, d, C-5 CH), 5.62 (1H, d, C-2 CH), 5.70 (2H, m, C-13 and C-3' CH), 6.22 (1H, s, C-10 CH), 6.74 (2H, d, MeOPh o-CH), 7.09-7.60 (23H, m, Ph), 7.80 and 8.09 (each 2H, d, Bz o-CH); MS (FAB) 1148 (M + Na)⁺, 1164 (M + K)⁺.

EXAMPLE 53Preparation of MC-Phe-N'-Mtr-Lys-PABC-7-Taxol-2'-Mtr (54)

35 2'-Methoxytrityl-taxol (53) (218.8 mg, 0.194 mmoles) in dry CH₂Cl₂ (3 ml) under argon at about 0°C was treated with DIEA (34 μ l, 1 equiv.), pyridine (15.7 μ l, 1 equiv.) and then diphosgene (12 μ l, 0.5 equiv.). The ice bath was removed and the mixture was stirred at room temperature for about 1 hour and then recooled to about 0 °C. MC-Phe-N'-Mtr-Lys-PAB-OH (47) (167.9 mg, 1 equiv.) was flushed with dry CH₂Cl₂ (6 ml), dried in vacuo and then dissolved in dry CH₂Cl₂ (2ml) and DIEA (34 μ l, 1 equiv.). This solution was added via syringe to the crude chloroformate at about 0 °C. After about 10 minutes the ice bath was removed and the mixture was stirred at room temperature for about 18 hours. The mixture was diluted with ethyl acetate and washed with pH 5 buffer, water and brine, dried and evaporated. The residue was chromatographed on silica, eluting with 1) 2:1 CH₂Cl₂/ethyl acetate, 2) 1:1 ethyl acetate/CH₂Cl₂, 3) 4:1 ethyl acetate/CH₂Cl₂ and 4) ethyl acetate, to give the product as a colorless glass (237.9 mg, 61%), along with unreacted starting materials. ¹H NMR (CDCl₃) δ 1.13, 1.16 and 1.57 (each 3H, s, C-16, C-17 and C-19 CH₃), 1.10-1.80 (12H, m, Lys and caproyl CH₂), 1.88 and 2.61 (each 1H, m, C-6 CH₂), 1.78 (3H, s, C-18 CH₃), 2.10 (4H, m, Lys N-CH₂ and caproyl CO-CH₂), 2.17 and 2.29 (each 3H, s, Ac CH₃), 3.06 (2H, m, Phe CH₂), 3.42 (2H, t, caproyl N-CH₂), 3.75 and 3.78 (each 3H, s, O-CH₃), 3.82 (1H, m, C-3 CH), 4.21 (2H, ABq, C-20 CH₂), 4.42 and 4.70 (each 1H, q, Phe and Lys CO-CH), 4.62 (1H, d, C-2' CH), 4.93 (1H, d, C-5 CH), 5.19 (2H, q, PAB CH₂), 5.59 (1H, m, C-7 CH), 5.62 (1H, d, C-2 CH), 5.72 (2H, m, C-3' CH and C-13 CH), 6.17 and 6.60 (each 1H, brd, amide NH), 6.32 (1H, s, C-10 CH), 6.64 (2H, s, M CH), 6.77 (4H, m, MeOPh o-CH), 7.05-7.62 (44H, m, Ph), 7.80 and 8.06 (each 2H, d, Bz o-CH), 8.37 (1H, brs, PAB NH).

EXAMPLE 54Preparation of MC-Phe-Lys-PABC-7-Taxol • CICH₂CO₂H (55)

5 A stirred solution of MC-Phe-N'-Mtr-Lys-PABC-7-Taxol-2'-Mtr 54 (194.8 mg, 0.097 mmoles) in CH₂Cl₂ - (4.5 ml) and anisole (1.05 ml, 100 equiv.) was treated with chloroacetic acid (1M in CH₂Cl₂, 0.97 ml, 10 equiv.). After about 4 hours ether (25 ml) was added. The resulting solid was collected by filtration and washed with ether (142.0 mg, 94%). ¹H NMR (CDCl₃) δ 1.13, 1.20 and 1.72 (each 3H, s, C-16, C-17 and C-19 CH₃), 1.10-1.90 (12H, m, Lys and caproyl CH₂), 2.13 and 2.33 (each 3H, s, Ac CH₃), 2.96 (2H, m, ¹H₃N-CH₂), 3.05 (2H, m, Phe CH₂), 3.38 (2H, m, caproyl N-CH₂), 3.86 (1H, d, C-3 CH), 4.21 (2H, m, C-20 CH₂), 4.50 and 4.61 (each 1H, m, Phe and Lys CO-CH), 4.77 (1H, brs, C-2' CH), 4.91 (1H, d, C-5 CH), 5.10 (2H, m, PAB CH₂), 5.42 (1H, m, C-7 CH), 5.64 (1H, d, C-2 CH), 5.71 (1H, m, C-3' CH), 6.11 (1H, m, C-13 CH), 6.30 (1H, s, C-13 CH), 6.73 (2H, s, M CH), 7.00-8.20 (24H, m, Ph); HPLC (C-18, 15 cm column, 7:3 acetonitrile/50 mM triethylammonium formate buffer (pH 2.8), 1 ml/min., 250nm): single peak, retention time 2.91 min.; MS (FAB) 1471.6 (MH⁺), 1509.5 (M + Na)⁺, 1511.8 (M + K)⁺.

EXAMPLE 55Preparation of Fmoc-Val-NHS (56)

20 Fmoc-Val (5.060 g, 14.91 mmoles) and NHS (1.72 g, 1 equiv.) in THF (50 ml) at about 0 °C were treated with DCC (3.080 g, 1 equiv.). The mixture was stirred at room temperature for about 16 hours and then the solid DCU by-product was filtered off and washed with THF. The solvent was removed on the rotovap and the resulting colorless, glassy solid was used without purification in the next step.

EXAMPLE 56Preparation of Fmoc-Val-Cit (57)

30 Fmoc-Val-NHS (56) (14.91 mmoles) in DME (40 ml) was added to a solution of L-citrulline (2.743 g, 1.05 equiv.) and NaHCO₃ (1.315 g, 1.05 equiv.) in water (40 ml). THF (20 ml) was added to aid solubility, and the mixture was stirred at room temperature for about 16 hours. Aqueous citric acid (15%, 75 ml) was added and the mixture was extracted with 10% isopropanol/ethyl acetate (2 x 100 ml). The solid product began to precipitate but remained with the organic layer. The suspension was washed with water (2 x 150 ml) and the solvents were evaporated. The resulting white solid was dried in vacuo for about 5 hours and then treated with ether (80 ml). After sonication and trituration the white solid product was collected by filtration (5.8007 g, 78%). ¹H-NMR (DMSO-d₆) δ 0.87 (6H, q, Val CH₃), 1.40, 1.59 and 1.69 (4H, m, Cit CH₂), 1.97 (1H, m, Val CH₃-CH), 2.94 (2H, q, Cit N-CH₂), 3.92 (1H, t, Fmoc CH), 4.10-4.35 (2H, m, Val and Cit CO-CH), 4.23 (2H, t, Fmoc CH₂), 5.37 (2H, brs, Cit NH₂), 5.92 (1H, t, Cit NH), 7.28-7.90 (8H, m, Ph), 8.15 (1H, d, amide NH); MS (FAB) 497 (MH⁺), 519 (M + Na)⁺, 535 (M + K)⁺; Accurate mass calc. for C₂₆H₃₃N₄O₆: 497.2400; found: 497.2394;

Anal. calc. for C ₂₆ H ₃₂ N ₄ O ₆ :			
	C-62.89, Found: C-62.92,	H-6.50, H-6.67,	N-11.28; N-11.07.

EXAMPLE 57Preparation of Fmoc-Val-Cit-PAB-OH (58)

50 Fmoc-Val-Cit (57) (1.0443 g, 2.103 mmoles) and p-aminobenzyl alcohol (518.0 mg, 2 equiv.) in 2:1 CH₂Cl₂/methanol (35 ml) were treated with EEDQ (1.0402 g, 2 equiv.). The mixture was stirred in the dark at room temperature for 1.5 days. The solvents were removed on the rotovap (bath t mp. about 40 °C) and the white solid residue was triturated with ether (75 ml). The resulting suspension was sonicated for about 5 minutes and then left to stand for about 30 minutes. The solid was collected by filtration and washed repeatedly with ether (1.0070 g, 80%). ¹H-NMR (DMSO-d₆) δ 0.88 (6H, t, Val CH₃), 1.41 and 1.65 (4H, m,

Cit CH₂), 2.00 (m, Val CH₃-CH), 2.99 (2H, m, Cit N-CH₂), 3.92 (1H, t, Fmoc CH), 4.24 (2H, d, Fmoc CH₂), 4.19-4.50 (2H, m, Val and Cit CO-CH), 4.43 (2H, d, PAB CH₂), 5.11 (1H, t, PAB OH), 5.42 (2H, brs, Cit NH₂), 5.98 (1H, t, Cit NH), 7.15-7.92 (12H, m, Ph), 8.12 (1H, d, amide NH), 9.99 (1H, brs, PAB NH); MS (FAB) 602 (MH)⁺, 624 (M + Na)⁺, 640 (M + K)⁺; Accurate mass calc. for C₃₃H₄₀N₅O₆: 602.2979; found: 5 602.2977;

Anal. calc. for C ₃₃ H ₃₉ N ₅ O ₆ :			
	C-65.87, Found: C-65.61,	H-6.53, H-6.49,	N-11.64; N-11.73.
10			

EXAMPLE 58

15 Preparation of Val-Cit-PAB-OH (59)

Fmoc-Val-Cit-PAB-OH (58) (245.2 mg, 407.5 μ moles) in NMP (4 ml) at room temperature was treated with diethylamine (0.8 ml). The mixture was left to stand at room temperature for about 16 hours and then the solvents were removed on the rotovap (bath temp about 40 °C). The thick, oily residue was treated with CH₂Cl₂ (15 ml). With scraping and sonication the first-formed gum became a solid which was collected by filtration and washed with CH₂Cl₂ (141.6 mg, 92%). ¹H-NMR (DMSO-d₆) δ 0.82 (6H, 2 x d, Val CH₃), 1.39, 20 1.59 and 1.66 (4H, m, Cit CH₂), 1.92 (1H, m, Val CH₃-CH), 2.98 (1H, m, Val CO-CH), 3.03 (2H, d, Val NH₂), 4.45 (2H, d, PAB CH₂), 4.48 (1H, m, Cit CO-CH), 5.10 (1H, brt, PAB OH), 5.41 (2H, brs, Cit NH₂), 5.99 (1H, brt, Cit NH), 7.21 and 7.52 (each 2H, d, PAB Ph), 8.12 (1H, brd, amide NH), 10.03 (1H, brs, PAB NH); MS (FAB) 380 (MH)⁺, 402 (M + Na)⁺, 418 (M + K)⁺.

EXAMPLE 59

30 Preparation of MC-Val-Cit-PAB-OH (60)

Val-Cit-PAB-OH (59) (136.8 mg, 360.5 μ moles) and MC-NHS (122.3 mg, 1.1 equiv.) in NMP (5 ml) at room temperature were left to stand for about 16 hours. The NMP was removed on the rotovap (bath temp. about 40 °C) and the thick, oily residue was triturated with ether (20 ml). The solid product was collected by filtration and washed repeatedly with ether (205.7 mg, 99.6%). ¹H-NMR (DMSO-d₆) δ 0.82 (6H, ABq, Val CH₃), 1.10-1.90 (10H, m, Cit and caproyl CH₂), 1.92 (1H, m, Val CH₃-CH), 2.16 (2H, t, caproyl CO-CH₂), 2.98 (2H, m, Cit N-CH₂), 3.33 (2H, t, M-CH₂), 4.19 (1H, t, Val CO-CH), 4.38 (1H, m, Cit CO-CH), 4.42 (2H, brd, PAB CH₂), 5.10 (1H, brt, PAB OH), 5.42 (2H, brs, Cit NH₂), 5.97 (1H, brt, Cit NH), 6.99 (2H, s, M CH), 7.21 and 7.52 (each 2H, d, PAB Ph), 7.82 and 8.07 (each 1H, d, amide NH), 9.90 (1H, brs, PAB NH); MS (FAB) 573 (MH)⁺, 595 (M + Na)⁺, 611 (M + K)⁺; Accurate mass calc. for C₂₈H₄₁N₆O₇: 573.3037; found: 40 573.3016.

EXAMPLE 60

45 Preparation of MC-Val-Cit-PABC-PNP (61)

MC-Val-Cit-PAB-OH (60) (112.4 mg, 196.3 μ moles) under argon at room temperature was dissolved in dry pyridine (3 ml). The solution was cooled to about 0 °C and p-nitrophenyl chloroformate (119 mg, 3 equiv.) in CH₂Cl₂ (2 ml) was added all at once. After about 10 minutes at about 0 °C the ice bath was removed and the mixture was stirred at room temperature for about 2 hours. Ethyl acetate (50 ml) and 50 15% citric acid (75 ml) were added. The organic phase was washed with more citric acid, water and brine, dried and evaporated to give a light-yellow gum. This was chromatographed on silica, eluting with 1) 20:1 and 2) 15:1 CH₂Cl₂/methanol, to give the product as a white solid (21.5 mg, 15%). ¹H-NMR (CDCl₃/CD₃OD) δ 0.90 (6H, d, Val CH₃), 1.16-1.95 (10H, m, Cit and caproyl CH₂), 2.12 (1H, m, Val CH₃-CH), 2.23 (2H, t, caproyl CO-CH₂), 3.17 (2H, m, Cit N-CH₂), 3.48 (2H, t, M-CH₂), 4.20 (1H, m, Val CO-CH), 4.59 (1H, m, Cit CO-CH), 5.22 (2H, s, PAB CH₂), 6.66 (2H, s, M CH), 6.91 and 7.79 (each 1H, d, amide NH), 7.34 and 7.60 (each 2H, d, PAB Ph), 7.34 and 8.23 (each 2H, d, PNP Ph), 9.49 (1H, brs, PAB NH); MS (FAB) 738 (MH)⁺, 760 (M + Na)⁺, 776 (M + K)⁺.

EXAMPLE 61Preparation of MC-Val-Cit-PABC-DOX (62)

5 MC-Val-Cit-PABC-PNP (61) (21.2 mg, 28.7 μ moles) and DOX-HCl (18.3 mg, 1.1 equiv.) in NMP (1.5 ml) at room temperature were treated with diisopropylethylamine (5.5 μ l, 1.1 equiv.). The mixture was left to stand in the dark at room temperature for 2 days and then CH_2Cl_2 (25 ml) was added. A fine precipitate formed. The suspension was stored in the freezer overnight and then the orange solid was collected by filtration and washed with CH_2Cl_2 . TLC showed some product remaining in the mother liquors along with most of the close-moving impurities. The crude solid was chromatographed on silica, eluting with 1) 15:1, 2) 10:1 and 3) 5:1 CH_2Cl_2 /methanol (the sample was loaded in a minimum amount of 2:1 CH_2Cl_2 /methanol), to give the product as an orange solid (22.4 mg, 68%). $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$) δ 0.83 (6H, d, Val CH_3), 1.18 (3H, d, sugar CH_3), 1.20-1.86 (12H, m, Cit and caproyl CH_2 , D-ring CH_2), 1.93 (1H, m, Val $\text{CH}_3\text{-CH}$), 2.12 (2H, m, D-ring CH_2), 2.17 (2H, t, caproyl CO-CH_2), 2.90-3.20 (4H, q and m, sugar CH_2 and Cit N-CH_2), 3.39 (2H, t, M- CH_2), 3.50 (1H, brs, HO- CH), 3.98 (3H, s, O- CH_3), 4.02 (1H, m, Val CO-CH), 4.05 (1H, m, sugar $\text{CH}_3\text{-CH}$), 4.46 (1H, m, Cit CO-CH), 4.68 (2H, s, CO- $\text{CH}_2\text{-OH}$), 4.88 (2H, q, PAB CH_2), 5.16 (1H, brs, anomeric CH), 5.39 (1H, brs, DOX Ph-CH), 6.62 (2H, s, M CH), 7.13 and 7.42 (each 2H, d, PAB Ph), 7.32, 7.71 and 7.92 (each 1H; d, t and d; DOX Ph); MS (FAB) 1141 (M) $^+$, 1164.6 (M+Na) $^+$, 1180 (M+K) $^+$; Accurate mass calc. for $\text{C}_{56}\text{H}_{67}\text{N}_7\text{O}_{19}\text{Na}$: 1164.4389; found: 1164.4363.

20

EXAMPLE 62Preparation of N-Boc-aminocaproic acid (63)

25 6-Aminocaproic acid (5.2331 g, 39.89 mmoles) and NaHCO_3 (3.3514 g, 1 equiv.) in water (50 ml) were treated with d-t-butyl dicarbonate (9.58 g, 1.1 equiv.). The mixture was stirred at room temperature overnight and then water (150 ml) and sat. NaHCO_3 (5 ml) were added. The solution was extracted with ether (100 ml) and then solid citric acid (10 g) was added, giving an oily suspension. This was extracted with Ethyl acetate (3 x). The combined organic phases were washed with water and brine, dried and evaporated to give a colorless oil which solidified under vacuum (9.23 g, quant.). $^1\text{H-NMR}$ (DMSO-d_6) δ 1.10-1.55 (6H, m, caproyl CH_2), 1.33 (9H, s, CH_3), 2.28 (2H, m, CO- CH_2), 2.88 (2H, m, N- CH_2), 6.77 (1H, m, NH); MS (DCI) 232 (MH) $^+$, 176 (MH- C_4H_9) $^+$.

35

Anal. calc. for $\text{C}_{11}\text{H}_{21}\text{NO}_4$:			
	C-57.12, Found: C-57.11,	H-9.15, H-9.22,	N-6.06; N-6.09.

40

EXAMPLE 63Preparation of Boc-NH-C-NHS (64)

45 N-Boc-aminocaproic acid (63) (9.23 g, 39.9 mmoles) and NHS (5.05 g, 1.1 equiv.) in THF (75 ml) at room temperature were treated with DCC (9.05 g, 1.1 equiv.). The mixture was stirred at room temperature for about 16 hours and then the solid DCU by-product was filtered off. The filtrate was evaporated to give a thick oil which was dissolved in CH_2Cl_2 (150 ml). After standing for about 1 hour more DCU was filtered off. The filtrate was again evaporated and the thick oily residue dried in vacuo upon which it gradually solidified. The product was used without further purification (13.052 g, 99.6%).

50

EXAMPLE 64Preparation of Boc-NH-C-Phe (65)

55 A solution of Boc-NH-C-NHS (64) (12.52 g, 38.13 mmoles) in DME (100 ml) was added to a solution of L-Phe (6.930 g, 1.1 equiv.) and NaHCO_3 (3.524 g, 1.1 equiv.) in water (100 ml) at room temperature. THF (30 ml) was added to increase solubility. The mixture was stirred at room temperature for about 16 hours and then 15% citric acid (100 ml) was added. The suspension was extracted with 10% isopropanol/ethyl

acetate (3 x 80 ml) and the combined organic phases were washed with water and brine, dried and evaporated to give a white solid. This was triturated with ether and the resulting white solid was collected by filtration and washed with ether (12.122 g, 84%). $^1\text{H-NMR}$ (DMSO-d₆) δ 1.09 and 1.30 (6H, m, caproyl CH₂), 1.38 (9H, s, CH₃), 1.80-2.25 (2H, m, CO-CH₂), 2.82 (4H, m, Phe CH₂ and N-CH₂), 4.52 (1H, m, CO-CH), 5.73 (1H, m, NH), 7.20 (5H, m, Ph); MS (DCI) 379 (MH)⁺, 323 (MH-C₄H₉)⁺, 279 (MH-C₅H₉O₂)⁺;

<u>Anal. calc. for C₂₀H₃₀N₂O₅:</u>			
	C-63.47, Found: C-63.37,	H-7.99, H-8.05,	N-7.40; N-7.76.

EXAMPLE 65

15 Preparation of Boc-NH-C-Phe-NHS (66)

Boc-NH-C-Phe (65) (11.527 g, 30.46 mmoles) and NHS (3.86 g, 1.1 equiv.) in THF (100 ml) at about 0°C were treated with DCC (6.913 g, 1.1 equiv.). The mixture was stirred at room temperature for about 16 hours and worked up as described above for Boc-NH-C-NHS (64) to give the product as a colorless glass which was used without further purification (14.369 g, 99.2%).

EXAMPLE 66

25 Preparation of Boc-NH-C-Phe-N'-Fmoc-Lys (67)

Boc-NH-C-Phe-NHS (66) (14.369 g, 30.22 mmoles) in DME (100 ml) was added to a solution of N'-Fmoc-Lys (11.222 g, 1 equiv.) and NaHCO₃ (2.560 g, 1 equiv.) in water (50 ml) and DME (50 ml). The mixture was stirred vigorously at room temperature for about 16 hours and then 15% citric acid (150 ml) and 10% isopropanol/ethyl acetate (250 ml) were added. The aqueous phase was extracted with more 10% isopropanol/ethyl acetate (2 x 100 ml). The combined organic phases were washed with water and brine, dried and evaporated to give an off-white solid. This was triturated with ether and the white, solid product was collected by filtration and washed with ether (17.842 g, 81%). $^1\text{H-NMR}$ (CDCl₃/CD₃OD) δ 1.00-1.92 (12H, m, Lys and caproyl CH₂), 1.42 (9H, s, CH₃), 2.09 (2H, m, CO-CH₂), 2.96 (2H, m, Phe CH₂), 3.10 (2H, m, caproyl N-CH₂), 3.31 (2H, m, Lys N-CH₂), 4.18 (1H, t, Fmoc CH), 4.37 (2H, d, Fmoc CH₂), 4.46 and 4.71 (each 1H, m, Phe and Lys CO-CH), 7.10-7.80 (13H, m, Ph); MS (FAB) 729 (MH)⁺, 751 (M + Na)⁺, 767 (M + K)⁺;

<u>Anal. calc. for C₄₁H₅₂N₂O₈-H₂O:</u>			
	C-65.93, Found: C-66.07,	H-7.32, H-7.32,	N-7.66; N-7.66.

45 EXAMPLE 67

Preparation of Boc-NH-C-Phe-N'-Fmoc-Lys-PAB-OH (68)

Boc-NH-C-Phe-N'-Fmoc-Lys (67) (15.716 g, 21.56 mmoles) and p-aminobenzyl alcohol (3.983 g, 1.5 equiv.) in THF (100 ml) at room temperature were treated with EEDQ (8.000 g, 1.5 equiv.). The mixture was stirred at room temperature for about 16 hours and then evaporated to dryness (water bath temperature 30°C). The residue was triturated with ether (100 ml) and the white, solid product was collected by filtration and washed with ether (16.453 g, 92%). $^1\text{H-NMR}$ (DMSO-d₆) δ 0.90-1.80 (12H, m, Lys and caproyl CH₂), 1.35 (9H, s, CH₃), 2.00 (2H, t, CO-CH₂), 2.66-3.07 (6H, m, N-CH₂ and Phe CH₂), 4.19 (1H, m, Fmoc CH), 4.23 (2H, d, Fmoc CH₂), 4.36 and 4.58 (each 1H, m, Phe and Lys CO-CH), 4.41 (2H, s, PAB CH₂), 7.10-8.22 (17H, m, Ph), 9.94 (1H, brs, PAB NH); MS (FAB) 834 (MH)⁺, 856 (M + Na)⁺, 872 (M + K)⁺;

5

Anal. calc. for C ₄₈ H ₅₉ N ₅ O ₈ -1/2H ₂ O:			
	C-68.39, Found: C-68.18,	H-7.17, H-7.12,	N-8.31; N-8.42.

EXAMPLE 68Preparation of MC-NH-C-Phe-N'-Fmoc-Lys-PAB-OH (69)

Boc-NH-C-Phe-N'-Fmoc-Lys-PAB-OH (60) (2.1323 g, 2.860 mmoles) was dissolved in 2:1 CH₂Cl₂/TFA (30 ml). The mixture was sonicated at room temperature for about 15 minutes and then left to stand for about 1 hour. The solvents were evaporated and the residual brown oil was dried in vacuo for about 1 hour. Ether (75 ml) was added and the oil was scraped until it solidified. The solid was collected by filtration, washed with ether and dried in vacuo for several hours. It was then dissolved in 3:1 DME/water (40 ml) and treated with a solution of MC-NHS (788.2 mg, 1 equiv.) in DME (20 ml) and solid NaHCO₃ (540 mg, 2.5 equiv.). The mixture was stirred at room temperature for about 16 hours. As much DME as possible was removed on the rotovap (water bath temp. about 30 °C), leaving a gummy solid (which eventually solidified) in water. The solid was filtered, washed with water and dried in vacuo. It was then triturated with ether (25 ml) and the solid product was collected by filtration and washed with ether (1.4283 g, 60%). ¹H-NMR (CDCl₃/CD₃OD) δ 1.00-1.90 (18H, m, Lys and caproyl CH₂), 2.07 (4H, m, Phe CH₂ and CO-CH₂), 2.22 (2H, t, CO-CH₂), 3.05 (4H, m, Lys N-CH₂ and caproyl N-CH₂), 3.41 (2H, m, M-CH₂), 4.11 (1H, t, Fmoc CH), 4.28 (2H, d, Fmoc CH₂), 4.38 and 4.63 (each 1H, m, Phe and Lys CO-CH), 4.52 (2H, s, PAB CH₂), 5.61 (2H, s, M CH), 6.96-7.71 (17H, m, Ph); MS (FAB) 927.5 (MH)⁺, 949.3 (M + Na)⁺, 965.3 (M + K)⁺; Accurate mass calc. for C₅₃H₆₃N₆O₉: 927.4657; found: 927.4642.

EXAMPLE 69Preparation of MC-NH-C-Phe-N'-Fmoc-Lys-PABC-PNP (70)

MC-NH-C-Phe-N'-Fmoc-Lys-PAB-OH (69) (1.3783 g, 1.487 mmoles) and p-nitrophenyl chloroformate (449.5 mg, 1.5 equiv.) in CH₂Cl₂ (50 ml) at room temperature were treated with pyridine (0.18 ml, 1.5 equiv.). The suspension was sonicated at room temperature for about 30 minutes and then stirred for about 16 hours. More p-nitrophenyl chloroformate (150 mg, 0.5 equiv.) and pyridine (0.06 ml, 0.5 equiv.) were added and the mixture was again sonicated for about 30 minutes and stirred for about 4 hours. Workup as described above for MC-Val-Cit-PABC-PNP (61) gave the crude product as a gummy solid. This was chromatographed on silica, eluting with 1) 35:1, 2) 25:1 and 3) 20:1 CH₂Cl₂/methanol, to give the product as a pale-yellow, gummy solid (593.1 mg, 0.543 mmoles). ¹H-NMR (CDCl₃/CD₃OD) δ 1.10-1.95 (18H, m, Lys and caproyl CH₂), 2.12 (4H, m, caproyl CO-CH₂), 3.00 (2H, m, Phe CH₂), 3.11 (4H, m, Lys and caproyl N-CH₂), 3.44 (2H, t, M-CH₂), 4.13 (1H, t, Fmoc CH), 4.32 (2H, d, Fmoc CH₂), 4.39 and 4.63 (each 1H, m, Phe and Lys CO-CH), 5.18 (2H, s, PAB CH₂), 6.63 (2H, s, M CH), 7.00-8.25 (21H, m, Ph); MS (FAB): 1114 (M + Na)⁺, 1130 (M + K)⁺; Accurate mass calc. for C₆₀H₆₆N₇O₁₃: 1092.4719; found: 1092.4680.

EXAMPLE 70Preparation of MC-NH-C-Phe-N'-Fmoc-Lys-PABC-DOX (71)

MC-NH-C-Phe-N'-Fmoc-Lys-PABC-PNP (70) (382.8 mg, 0.350 mmoles) and DOX-HCl (213 mg, 1.05 equiv.) in NMP (16 ml) were treated with diisopropylethylamine (61 μ l, 1 equiv.). The mixture was allowed to stand in the dark for 2 days. Workup as described above for MC-Val-Cit-PABC-DOX (62) gave the product as an orange glass (293.1 mg, 56%). ¹H-NMR (CDCl₃/CD₃OD) δ 1.00-1.85 (20H, m, Lys and caproyl CH₂, D-ring CH₂), 1.21 (3H, d, sugar CH₃), 2.09 (4H, m, caproyl CO-CH₂), 2.17 (2H, m, D-ring CH₂), 2.80-3.27 (8H, m, Lys and caproyl N-CH₂, sugar CH₂, Phe CH₂), 3.40 (2H, t, M-CH₂), 3.53 (1H, brs, HO-CH), 3.78 (1H, m, sugar N-CH), 3.99 (3H, s, O-CH₃), 4.11 (2H, t, Fmoc CH and sugar CH₃-CH), 4.29 (2H, d, Fmoc CH₂), 4.33 and 4.57 (each 1H, m, Phe and Lys CO-CH), 4.71 (2H, s, CO-CH₂-OH), 4.89 (2H, q, PAB CH₂), 5.20 (1H, brs, anomeric CH), 5.42 (1H, brs, DOX Ph-CH), 6.60 (2H, s, M CH), 6.90-8.00 (20H, m, Ph); MS (FAB) 1519 (M + Na)⁺, 1534 (M + K)⁺; Accurate mass calc. for C₈₁H₈₉N₇O₂₁Na: 1518.6009; found: 1518.5962.

EXAMPLE 71Preparation of MC-NH-C-Phe-Lys-PABC-DOX·HCl (72)

5 MC-NH-C-Phe-N'-Fmoc-Lys-PABC-DOX (71) (95.2 mg, 63.6 μ moles) in NMP (0.3 ml) was diluted with THF (10 ml) and then, with stirring, treated with 2% DBU in THF (10 ml). After about 45 seconds ether (40 ml) was added and the resulting blue solid was collected by filtration and washed with ether. The solid was resuspended in ether (10 ml) and treated with 1M HCl in ether (10 ml). After several minutes the orange solid was filtered off, washed repeatedly with ether and triturated with CH_2Cl_2 (25 ml). The resulting orange-red solid was collected by filtration and chromatographed on LH-20 lipophilic sephadex, eluting with 1:1 CH_2Cl_2 /methanol. The product-containing fractions were combined and re-chromatographed on LH-20, eluting with methanol, to give the product as an orange glass, with minor contaminants as shown by HPLC (40.2 mg, 48.2%). $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$) δ (selected peaks) 1.00-1.95 (23H, m, sugar CH_3 , Lys and caproyl CH_2 , D-ring CH_2), 2.00-2.40 (6H, m, caproyl CO- CH_2 and D-ring CH_2), 2.96 (2H, m, $^+\text{H}_3\text{N}-\text{CH}_2$), 4.05 (3H, s, O- CH_3), 4.72 (2H, s, CO- CH_2-OH), 4.93 (2H, brs, PAB CH_2), 5.17 (1H, brs, anomeric CH), 5.42 (1H, brs, DOX Ph-CH), 6.63 (2H, brs, M CH), 6.90-8.20 (12H, m, Ph).

EXAMPLE 72Preparation of Fmoc-Phe-N'-Mtr-Lys-NHS (73)

20 A stirred mixture of Fmoc-Phe-N'-Mtr-Lys (44) (1.8873 g, 2.40 mmoles) and NHS (303.2 mg, 1.1 equiv.) in CH_2Cl_2 (40 ml) at about 0 °C was treated with DCC (543.6 mg, 1.1 equiv.). After about 24 hours at room temperature the DCU was filtered off and the filtrate evaporated and the residue taken up in ethyl acetate. 25 This was washed with water (2x) and brine, dried and evaporated. The residue was chromatographed on silica, eluting with 1:1 ethyl acetate/hexane. Much of the product decomposed on the column (472.5 mg, 22%). $^1\text{H-NMR}$ (CDCl_3) δ 1.00-1.98 (6H, m, CH_2), 2.01 (2H, t, N- CH_2), 2.77 (4H, brs, NHS CH_2), 3.09 (2H, m, Phe CH_2), 3.76 (3H, s, O- CH_3), 4.10-4.51 (4H, m, Fmoc CH_2 and CH, Phe CO-CH), 4.83 (1H, m, Lys CO-CH), 5.48 and 6.41 (each 1H, m, NH), 6.79 (2H, d, MeOPh o-CH), 7.06-7.80 (25H, m, Ph).

EXAMPLE 73Preparation of Fmoc-Phe-N'-Mtr-Lys-GABA (74)

35 A solution of Fmoc-Phe-N'-Mtr-Lys-NHS (73) (472.5 mg, 0.534 mmoles) in DME (25 ml) was added to a stirred solution of GABA (83 mg, 1.5 equiv.) and NaHCO_3 (67 mg, 1.5 equiv.) in water (15 ml) at room temperature. After 16 hours at room temperature as much DME as possible was removed on the rotovap and the resulting suspension was partitioned between ethyl acetate and pH 5 buffer. The organic phase was washed with water and brine, dried and evaporated. The residue was triturated with ether and the resulting 40 white solid collected by filtration (387.0 mg, 83%). $^1\text{H-NMR}$ (CDCl_3) δ 0.96-1.99 (8H, m, CH_2), 2.10-2.42 (4H, m, Lys N- CH_2 and CO- CH_2), 3.03 (2H, m, Phe CH_2), 3.22 (2H, m, GABA N- CH_2), 4.03-4.66 (5H, m, Fmoc CH_2 and CH, CO-CH), 6.78 (2H, d, MeOPh o-CH), 7.00-7.77 (25H, m, Ph); MS (FAB) 895 ($M + \text{Na}$) $^+$, 911 ($M + \text{K}$) $^+$.

EXAMPLE 74Preparation of Fmoc-Phe-N'-Mtr-Lys-GABA-MMC (75)

50 A stirred mixture of Fmoc-Phe-N'-Mtr-Lys-GABA (74) (296.9 mg, 0.340 mmoles), HOBT (46 mg, 1 equiv.) and MMC (119.4 mg, 1.05 equiv.) in NMP (3 ml) and CH_2Cl_2 (3 ml) at room temperature was treated with DCC (77.2 mg, 1.1 equiv.). After about 14 hours at room temperature ethyl acetate was added and the solution was washed with water (3x) and brine, dried and evaporated. The residue was chromatographed on silica, eluting with 25:1 CH_2Cl_2 /methanol, to give the product as a purple glass (303.1 mg, 75%). $^1\text{H-NMR}$ (CDCl_3) δ 0.97-1.90 (8H, m, CH_2), 1.71 (3H, s, MMC CH_3), 2.08 (2H, m, Lys N- CH_2), 2.46 (2H, m, CO- CH_2), 2.99 (2H, m, Phe CH_2), 3.12 (2H, m, GABA N- CH_2), 3.20 (3H, s, MMC O- CH_3), 3.28-3.55 (3H, m, C-1, C-2 and C-3 CH), 3.68 (1H, ABq, C-9 CH), 3.73 (3H, s, Mtr O- CH_3), 4.04-4.51 and 4.64 (7H, m, Fmoc CH_2 and CH, C-10 CH_2 , CO-CH), 5.14 (2H, br, NH₂), 5.38, 5.49, 5.70 and 6.67 (each 1H, br, NH), 6.79 (2H, d, MeOPh o-CH), 7.03-7.78 (25H, m, Ph); MS (FAB) 1189.8 (MH) $^+$, 1211 ($M + \text{Na}$) $^+$, 1227.5 ($M + \text{K}$) $^+$.

EXAMPLE 75Preparation of Phe-N^t-Mtr-Lys-GABA-MMC (76)

5 Fmoc-Phe-N^t-Mtr-Lys-GABA-MMC (75) (236.1 mg, 0.198 mmoles) in CH₂Cl₂ (2 ml) at room temperature was treated with diethylamine (2 ml). After about 3 hours the solvents were evaporated and the residue was flushed with CH₂Cl₂ (10 ml). The residue was chromatographed on silica, eluting with 1) 25:1 and 2) 15:1 CH₂Cl₂/methanol, to give the product as a purple glass (157.4 mg, 82%). ¹H-NMR (CDCl₃) δ 1.15-1.83 (8H, m, CH₂), 1.77 (3H, s, MMC CH₃), 2.10 (2H, t, Lys N-CH₂), 2.46 (2H, m, CO-CH₂), 2.69 and 3.21 (each 1H, ABq, Phe CH₂), 3.19 (3H, s, MMC O-CH₃), 3.20-3.53 (5H, m, GABA N-CH₂, C-1, C-2 and C-3 CH), 3.48 (2H, brs, NH₂), 3.68 (2H, m, C-9 CH and Phe CO-CH), 3.76 (3H, s, Mtr O-CH₃), 4.09 and 4.82 (each 1H, t and ABq, C-10 CH₂), 4.29 (1H, m, Lys CO-CH), 4.41 (1H, d, C-3 CH), 5.29 (2H, brs, NH₂), 6.60 (1H, brt, GABA NH), 6.79 (2H, d, MeOPh o-CH), 7.10-7.48 (17H, m, Ph), 7.72 (1H, d, amide NH); MS (FAB) 967.4 (MH)⁺, 989.2 (M + Na)⁺, 1005.3 (M + K)⁺.

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EXAMPLE 76Preparation of MC-Phe-N^t-Mtr-Lys-GABA-MMC (77)

20 A solution of Phe-N^t-Mtr-Lys-GABA-MMC (76) (108.9 mg, 0.113 mmoles) in CH₂Cl₂ (15 ml) was added to MC-NHS (0.124 mmoles). The mixture was stirred at room temperature for 3 days and then the solvent was evaporated. The residue was chromatographed on silica, eluting with 1) 20:1 and 2) 15:1 CH₂Cl₂/methanol, to give the product as a purple glass (75.8 mg, 58%). ¹H-NMR (CDCl₃) δ 1.05-1.90 (14H, m, CH₂), 1.76 (3H, s, MMC CH₃), 2.07 (4H, m, Lys N-CH₂ and caproyl CO-CH₂), 2.49 (2H, m, GABA CO-CH₂), 2.98 and 3.20 (each 1H, ABq, Phe CH₂), 3.19 (2H, m, GABA N-CH₂), 3.23 (3H, s, MMC O-CH₃), 3.33 (2H, t, M-CH₂), 3.20-3.53 (3H, m, C-1, C-2 and C-3 CH), 3.68 (1H, ABq, C-9 CH), 3.78 (3H, s, Mtr O-CH₃), 4.11 and 4.62 (each 1H, t and ABq, C-10 CH₂), 4.24 (1H, m, Lys CO-CH), 4.49 (1H, d, C-3 CH), 5.19 (2H, br, NH₂), 6.27 (1H, d, NH), 6.67 (2H, s, M CH), 6.72 (1H, brt, NH), 6.80 (2H, d, MeOPh o-CH), 7.10-7.47 (17H, m, Ph), 7.19 (1H, d, NH).

25

EXAMPLE 77Preparation of MC-Phe-Lys-GABA-MMC • ClCH₂CO₂H (78)

30 MC-Phe-N^t-Mtr-Lys-GABA-MMC (77) (43.2 mg, 37.2 μmoles) in CH₂Cl₂ (2 ml) was treated with anisole (0.405 ml, 100 equiv.) and chloroacetic acid (1M in CH₂Cl₂, 0.40 ml, 11 equiv.). After about 3 hours ether (5 ml) was added and the mixture was stored in the freezer for about 1 hour. The resulting solid was collected by filtration, washed with ether, and triturated with CH₂Cl₂ (36.1 mg, 99%). ¹H-NMR (CDCl₃/CD₃OD) δ 1.03-1.82 (8H, m, CH₂), 1.71 (3H, s, MMC CH₃), 2.08 (2H, t, caproyl CO-CH₂), 2.40 (2H, brt, GABA CO-CH₂), 2.83 (4H, m, GABA N-CH₂ and N^t-CH₂), 3.39 (2H, t, M-CH₂), 3.59 (1H, ABq, C-9 CH), 3.95 (1H, t, C-10 CH₂), 4.18 (1H, m, Lys CO-CH), 4.42 (1H, d, C-3 CH), 4.67 (2H, m, Phe CO-CH and C-10 CH₂), 6.63 (2H, s, M CH), 7.17 (5H, m, Ph); HPLC: (C-18, 15 cm column, 65:35 methanol/50 mM triethylammonium formate buffer (pH 2.8), 1 ml/min., 360 nm): single peak, retention time: 2.19 min.

35

EXAMPLE 78Preparation of Taxol-2'-ethyl carbonate-7-chloroformate (83)

40 A stirred solution of taxol-2'-ethyl carbonate (82) (154.2 mg, 0.1665 mmoles) in CH₂Cl₂ (3 ml) at 0 °C under argon was treated with pyridine (13.5 μl, 1 equiv.) and then diphosgene (10.0 μl, 0.5 equiv.). The ic bath was removed and the mixture was stirred at room temperature for one hour and then re-cooled to 0 °C and used immediately.

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EXAMPLE 79Preparation of MC-Phe-N'-Mtr-Lys-PABC-7-Taxol-2'-ethyl carbonate (84)

5 A solution of MC-Phe-N'-Mtr-Lys-PAB-OH (47) (143.9 mg, 0.1665 mmoles) in CH₂Cl₂ (4 ml) was added to the above solution of taxol-2'-ethyl carbonate-7-chloroformate (83) (0.1665 mmoles) at about 0°C. The ice bath was removed and the mixture was stirred at room temperature for about 3 hours. Ethyl acetate was then added and the solution was washed with pH 5 buffer, water, and brine, dried and evaporated to give a colorless glass which was chromatographed on silica, eluting with 1) 2:1 and 2) 1:1 CH₂Cl₂/ethyl acetate, to give the product as a colorless glass (251.0 mg, 83%). ¹H-NMR (CDCl₃) δ 1.16, 1.21 and 1.78 (each 3H, s, C-16, C-17 and C-19 CH₃), 1.10-1.90 (12H, m, Lys and caproyl CH₂), 1.31 (3H, t, ethyl CH₃), 1.91 and 2.60 (each 1H, m, C-6 CH₂), 2.04 (3H, s, C-18 CH₃), 2.12 (4H, t, Lys N-CH₂ and caproyl CO-CH₂), 2.18 and 2.48 (each 3H, s, Ac CH₃), 2.22 and 2.40 (each 1H, m, C-14 CH₂), 3.03 (2H, m, Phe CH₂), 3.42 (2H, t, caproyl N-CH₂), 3.97 (1H, d, C-3 CH), 4.29 (2H, m, C-20 CH₂), 4.21 (2H, q, ethyl CH₂), 4.46 and 4.72 (each 1H, m, Phe and Lys CO-CH), 4.96 (1H, d, C-5 CH), 5.16 (2H, q, PAB CH₂), 5.44 (1H, d, C-2' CH), 5.56 (1H, m, C-7 CH), 5.70 (1H, d, C-2 CH), 5.97 (1H, m, C-3' CH), 6.26 (1H, m, C-13 CH), 6.40 (1H, s, C-10 CH), 6.65 (2H, s, M CH), 6.78 (2H, d, MeOPh o-CH), 6.98 and 7.60 (each 1H, d, NH), 7.04-8.20 (31H, m, Ph), 8.38 (1H, brs, PAB NH); MS (FAB) 1837.2 (M + Na)⁺, 1853.5 (M + K)⁺.

EXAMPLE 80Preparation of MC-Phe-Lys-PABC-7-Taxol-2'-ethyl carbonate • ClCH₂CO₂H (85)

20 A stirred solution of MC-Phe-N'-Mtr-Lys-PABC-7-Taxol-2'-ethyl carbonate (84) (80.2 mg, 44.2 μmoles) in CH₂Cl₂ (3.5 ml) at room temperature was treated with anisole (0.48 ml, 100 equiv.) and chloroacetic acid (1M in CH₂Cl₂, 0.442 ml, 10 equiv.). After about 3 hours ether (15 ml) was added and the mixture was stored in the freezer for about 2 hours. The resulting white solid was collected by filtration and washed with ether (72.2 mg, 99%). ¹H-NMR (CDCl₃) δ 1.16, 1.20 and 1.80 (each 3H, s, C-16, C-17 and C-19 CH₃), 1.10-1.90 (12H, m, Lys and caproyl CH₂), 1.30 (3H, t, ethyl CH₃), 1.91 and 2.58 (each 1H, m, C-6 CH₂), 2.02 (3H, s, C-18 CH₃), 2.13 (2H, m, caproyl CO-CH₂), 2.17 and 2.45 (each 3H, s, Ac CH₃), 2.20 and 2.39 (each 1H, m, C-14 CH₂), 2.97 (2H, m, Lys N-CH₂), 3.01 (2H, m, Phe CH₂), 3.42 (2H, t, caproyl N-CH₂), 3.97 (1H, d, C-3 CH), 4.29 (4H, m, C-20 CH₂ and ethyl CH₂), 4.56 and 4.83 (each 1H, m, Phe and Lys CO-CH), 4.95 (1H, d, C-5 CH), 5.17 (2H, q, PAB CH₂), 5.42 (1H, d, C-2' CH), 5.54 (1H, m, C-7 CH), 5.69 (1H, d, C-2 CH), 5.97 (1H, m, C-3' CH), 6.29 (1H, m, C-13 CH), 6.41 (1H, s, C-10 CH), 6.66 (2H, s, M CH), 6.98 and 8.39 (each 1H, d, NH), 7.08-8.14 (19H, m, Ph), 9.25 (1H, brs, PAB NH).

EXAMPLE 81Conjugate Synthesis

25 A solution (10ml) of mAb BR96 (10.46 mg/ml, 6.54 × 10⁻⁵ M; concentration determined by UV absorption at 280 nm, 1 mg/ml of mAb equals 1.4 abs. units) in 0.125M potassium phosphate buffer was treated with a freshly prepared solution (0.523 ml) of 10mM dithiothreitol (DTT) at about 37°C for about 3 hours under nitrogen. The solution was transferred to an Amicon cell and was diafiltrated against phosphate buffered saline (PBS) until the effluent was free of SH groups (Ellman reagent). The mAb and SH group concentration was determined (10.11 mg/ml (6.32 × 10⁻⁵ M) and 4.48 × 10⁻⁴ M, respectively, representing a molar ratio (MR) of SH to mAb of 7.01). This solution was treated with MC-Phe-Lys-PABC-DOX (5 mg/ml, 4.77 × 10⁻³ M) in distilled water (1.2 ml), then left to stand overnight at about 4°C. The solution was transferred to a dialysis tube and dialysed 3 times against 1 L PBS for about 24 hours at about 4°C. The conjugate solution was filtered through a Millex-GV 0.22 μm filter unit (Millipore Corp.), and the filtrate was shaken gently for several hours with Bio-beads (Bio-Rad Laboratories) followed by another filtration through a Millivexx-GV unit. The concentration of DOX was determined from the UV absorbance at 495 nm ($\epsilon = 8030$, 283 μm, 164 μg/ml) and that of the mAb at 280 nm with a correction for DOX absorbance at 280 nm according to the formula:

55

$$\text{mAb (mg/ml)} = \frac{\text{A}_{280} - (0.724 \times \text{A}_{495})}{1.4}$$

where A is the observed absorbance at the noted wavelength.

Example 82

5 A solution of Phe-(N^t-MTR)Lys-PABC-DOX in an appropriate solvent is treated with an equivalent amount of N-Succinimidyl p-(iodoacetamido)benzoate. The solution is kept at about 30 °C for about 1 hour and then the solvent is evaporated under reduced pressure. The protecting group MTR is removed from the peptide in the usual manner and the iodoacetylated peptide is dissolved in water or an organic water miscible solvent to a known concentration. An appropriate amount of this solution is added to a solution of thiolated mAb BR96 in PBS to react with all thiol groups generated in the mAb. The solution is kept at about 4 °C for about one hour and then chromatographed over a size exclusion column to eliminate low molecular weight compounds from the conjugate. Finally the conjugate solution is shaken with a small amount of Bio-Beads for a few hours, then filtered through a 0.22 micron filter. The concentration of mAb and DOX is determined from their absorption at 280 and 495 nm, respectively and the MR of drug to mAb is calculated.

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Example 83

20 A solution of Phe-(N^t-MTR)Lys-PABC-DOX in an appropriate solvent is treated with an equivalent amount of N-Succinimidyl-3-(2-pyridynidithio)-propionate (SPDP). The solution is kept at about 30 °C for about 1 hour and then the solvent is evaporated under reduced pressure. The protecting group MTR is removed from the peptide in the usual manner and the peptide is dissolved in water or an organic water miscible solvent to a known concentration. An appropriate amount of this solution is added to a solution of thiolated mAb BR96 in PBS to react with all thiol groups generated in the mAb. The solution is kept at about 4 °C for about one hour and then chromatographed over a size exclusion column to eliminate low molecular weight compounds 25 from the conjugate. Finally the conjugate solution is shaken with a small amount of Bio-Beads for a few hours, then filtered through a 0.22 micron filter. The concentration of mAb and DOX is determined from their absorption at 280 and 495 nm, respectively and the MR of drug to mAb is calculated.

30 The invention has been described with reference to specific examples, materials and data. As one skilled in the art will appreciate, alternate means for using or preparing the various aspects of the invention may be available. Such alternate means are to be construed as included within the intent and spirit of the present invention as defined by the following claims.

Claims

35 1. A compound of the formula (I):



40

in which

L is a ligand;

A is a carboxylic acyl unit;

45

Y is an amino acid;

Z is an amino acid;

X and W are each a spacer;

50

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said functional group selected from a primary or secondary amine, hydroxyl, sulphydryl, carboxyl, aldehyde or a ketone;

n is an integer of 0 or 1; and

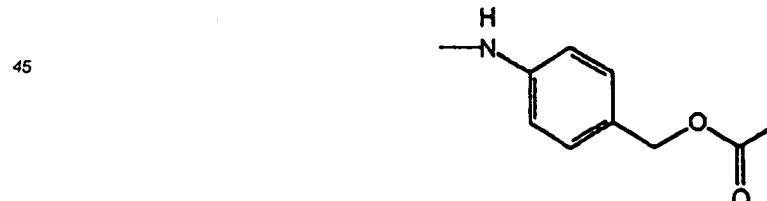
m is an integer of 1, 2, 3, 4, 5, or 6.

2. The compound of claim 1 wherein Y is alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan or prolin .

55 3. The compound of claim 1 wherein Z is lysine, lysine protected with acetyl or formyl, arginine, arginine protected with tosyl or nitro groups, histidin , ornithin , ornithine protected with acetyl or formyl, or

citrulline.

4. The compound of claim 1 wherein Y-Z is phenylalanine-lysine, valine-citrulline, or valine-lysine.
5. The compound of any of claims 1 through 4 wherein D is a cytotoxic drug.
6. The compound of claim 5 wherein D is the amino containing cytotoxic moiety mitomycin C, mitomycin A, daunorubicin, doxorubicin, N-(5,5-diacetoxypentyl)doxorubicin, aminopterin, actinomycin, bleomycin, 9-amino camptothecin, N⁸-acetyl spermidine, 1-(2-chloroethyl)-1,2-dimethanesulfonyl hydrazine, tal-lysomycin, or derivatives thereof.
7. The compound of claim 5 wherein D is the hydroxyl containing cytotoxic drug moiety etoposide, camptothecin, taxol, esperamicin, 1,8-dihydroxy-bicyclo[7.3.1]trideca-4-ene-2,6-diene-13-one, anguidine, doxorubicin, morpholine-doxorubicin, N-(5-5-diacetoxypentyl) doxorubicin, vincristine, vinblastine or derivatives thereof.
8. The compound of claim 5 wherein D is the sulphydryl containing cytotoxic drug moiety esperamicin and 6-mercaptopurine, or derivatives thereof.
9. The compound of any of claims 1 through 4 wherein D is the carboxyl containing drug moiety methotrexate, camptothecin (ring-opened form of the lactone), butyric acid, retinoic acid, or derivatives thereof.
10. The compound of claim 5 wherein D is the aldehyde and ketone containing drug moiety which is an anthracycline.
11. The compound of any of claims 1 through 10 wherein L is an immunoglobulin, or an antigen binding fragment thereof.
12. The compound of claim 11 wherein L is BR96, BR64, L6, a relaxed BR96, a relaxed BR64, a relaxed L6, a chimeric BR96, a chimeric BR64, a chimeric L6, a relaxed chimeric BR96, a relaxed chimeric BR64, a relaxed chimeric L6, or an antigen binding fragments thereof.
13. The compound of any of claims 1 through 10 wherein L is an epidermal growth factor, bombesin, transferrin, gastrin, gastrin-releasing peptide, platelet-derived growth factor, IL-2, IL-6, TGF- α , VGF, insulin or insulin-like growth factor I or II.
14. The compound of any of claims 1 through 10 wherein L is a carbohydrate, lectin, or apoprotein of a low density lipoprotein.
15. The compound of any of claims 1 through 14, wherein X is a compound having the formula



16. The compound of claim 15 wherein X is p-aminobenzyl-carbamoyl.
17. The compound of any of claims 1 through 14 wherein X is a compound having the formula

-HN-R'-CO-

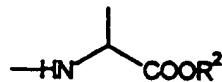
wherein R^1 is $\text{C}_1\text{-}\text{C}_5$ -alkylene.

18. The compound of claim 17 wherein X is derived from γ -aminobutyric acid, α,α -dimethyl- γ -aminobutyric acid, or β,β -dimethyl- γ -aminobutyric acid.

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19. The compound of any of claims 1 through 14 wherein X is a compound having the formula

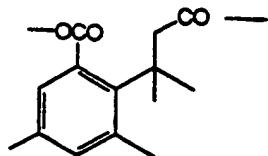
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wherein R^2 is H or $\text{C}_1\text{-}\text{C}_5$ alkyl.

15 20. The compound of any of claims 1 through 19 wherein W is a compound having the formula

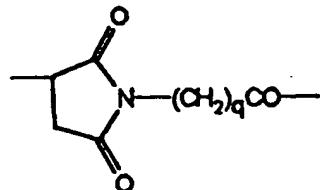
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21. The compound of any of claims 1 through 20 wherein A is a compound having the formula

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wherein q is an integer from 1-10.

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22. The compound of any of claims 1 through 20 wherein A is 4-(N-succinimidomethyl)cyclohexane-1-carbonyl, m-succinimidobenzoyl, 4-(p-succinimidophenyl)butryl, 4-(2-acetamido)benzoyl, 3-thio-propionyl, 4-(1-thioethyl)-benzoyl, or 6-(3-thiopropionylamido)-hexanoyl.

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23. The compound of claim 1 which is BR96-succinimidocaproyl-valine-lysine-p-aminobenzyl-carbamoyl-doxorubicin, BR96-succinimidocaproyl-valine-citrulline-p-aminobenzyl-carbamoyl-doxorubicin, BR96-succinimidocaproyl-phenylalanine-lysine-p-aminobenzyl-carbamoyl-2'-taxol, BR96-succinimidocaproyl-phenylalanine-lysine-p-aminobenzyl-carbamoyl-7-taxol, BR96-succinimidocaproyl-phenylalanine-lysine-p-aminobenzyl-carbamoyl-mitomycin C, or BR96-succinimidocaproyl-phenylalanine-lysine-gamma-aminobutyric acid-mitomycin C.

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24. A pharmaceutical composition comprising at least one compound of any of claims 1 through 23 and a pharmaceutically acceptable carrier.

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25. The use of at least one compound as defined in claim 1 to 23 for preparing a pharmaceutical composition for use in controlling the growth of undesirable cells said composition comprising at least one compound having a pharmaceutical ligand specific for the undesirable cell.

26. The use of claim 25 wherein the undesirable cells are cancer cells.

27. The method for preparation of maleimidocaproyl-phenylalanyl-lysine-p-aminobenzyl-carbamoyl-doxorubicin which comprises:

- (a) reacting N^a-9-fluorenylmethoxycarbamoyl-lysine with p-anisylidiphenylmethyl chloride, to give N^a-9-fluoromethoxycarbamoyl-N^a-p-methoxytrityl-lysine;
- 5 (b) removing the protecting group from N^a-9-fluorenylmethoxycarbamoyl-N^a-p-methoxytrityl-lysine by treating with a base to give N^a-p-methoxytrityl-lysine;
- (c) preparing 9-fluorenylmethoxycarbamoyl-phenylalanyl-N-hydroxysuccinimide by treating 9-fluorenylmethoxycarbamoyl-phenylalanine with N-hydroxysuccinimide in the presence of a condensing agent;
- 10 (d) coupling N^a-p-methoxytrityl-lysine and 9-fluorenylmethoxycarbamoyl-phenylalanyl-N-hydroxysuccinimide to give 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^a-p-methoxytrityl-lysine;
- (e) preparing 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl alcohol by treating 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^a-p-methoxytrityl-lysine with p-aminobenzyl alcohol;
- 15 (f) removing the protecting group from 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl alcohol by treating with a base to give phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl alcohol;
- (g) preparing maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl alcohol by treating phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl alcohol with maleimidocaproyl-N-hydroxysuccinimide in the presence of a base;
- 20 (h) treating maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl alcohol with excess bis-p-nitrophenyl carbonate to give maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl-p-nitrophenyl carbonate;
- (i) coupling maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl-p-nitrophenyl carbonate with doxorubicin to give maleimidocaproylphenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzylcarbamoyl-doxorubicin; and
- 25 (j) removing the lysine protecting group by treating with an acid in the presence of a cation scavenger to give maleimidocaproyl-phenylalanyl-lysyl-p-aminobenzylcarbamoyl-doxorubicin-dichloro acetic acid.

30 28. The method of claim 27 wherein the deprotection of maleimidocaproyl-phenylalanine-N^a-p-methoxytrityl-lysyl-p-aminobenzylcarbamoyl-doxorubicin comprises treating with ten equivalents of dichloroacetic acid and 100 equivalents of anisole in methylene chloride for one to three hours, followed by precipitating the product, maleimidocaproyl-phenylalanine-lysyl-p-aminobenzylcarbamoyl-doxorubicin-dichloroacetic acid with ethyl acetate.

35 29. An intermediate selected from maleimidocaproyl-phenylalanyl-lysyl-p-aminobenzylcarbamoyl-doxorubicin-dichloroacetic acid;

- maleimidocaproyl-valinyl-citrullyl-p-aminobenzylcarbamoyl-doxorubicin;
- 40 maleimidocaproyl-phenylalanyl-lysyl-p-aminobenzylcarbamoyl-7-taxol-chloroacetic acid;
- maleimidocaproyl-phenylalanyl-lysyl-p-aminobenzylcarbamoyl-7-taxol-2'-ethyl carbonate-chloroacetic acid;
- 45 maleimidocaproyl-phenylalanyl-lysyl-p-aminobenzylcarbamoyl-mitomycin C-chloroacetic acid;
- maleimidocaproyl-phenylalanyl-lysyl-γ-aminobutyryl-mitomycin C-chloroacetic acid;
- phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl alcohol;
- 50 maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl alcohol;
- maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl-p-nitrophenyl carbonate;
- maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzylcarbamoyl-doxorubicin;
- 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzyl alcohol;
- 55 valinyl-citrullyl-p-aminobenzyl alcohol;
- maleimidocaproyl-valinyl-citrullyl-p-aminobenzyl alcohol;
- maleimidocaproyl-valinyl-citrullyl-p-aminobenzyl-p-nitrophenyl carbonate;
- maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzylcarbamoyl-7-taxol-2'-p-methoxytrityl ether;
- 60 maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzylcarbamoyl-7-taxol-2'-thyl carbonate;
- maleimidocaproyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-p-aminobenzylcarbamoyl-mitomycin C;
- 9-fluorenylmethoxycarbamoyl-phenylalanyl-N^a-p-methoxytrityl-lysyl-γ-aminobutyric acid;

9-fluorenylmethoxycarbamoyl-phenylalanyl-N'-p-methoxytrityl-lysyl- γ -aminobutyryl-mitomycin C;
phenylalanyl-N'-p-methoxytrityl-lysyl- γ -aminobutyryl-mitomycin C and
maleimidocaproyl-phenylalanyl-N'-p-methoxytrityl-lysyl- γ -aminobutyryl-mitomycin C.

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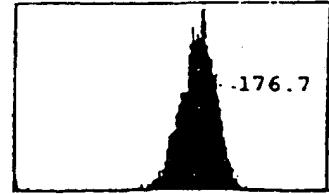
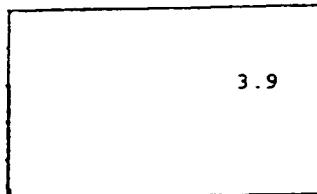
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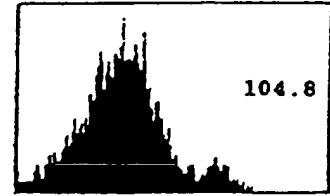
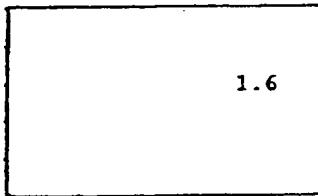
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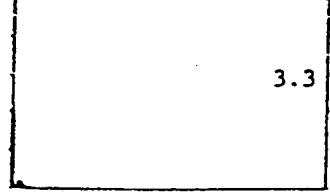
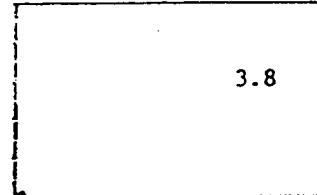
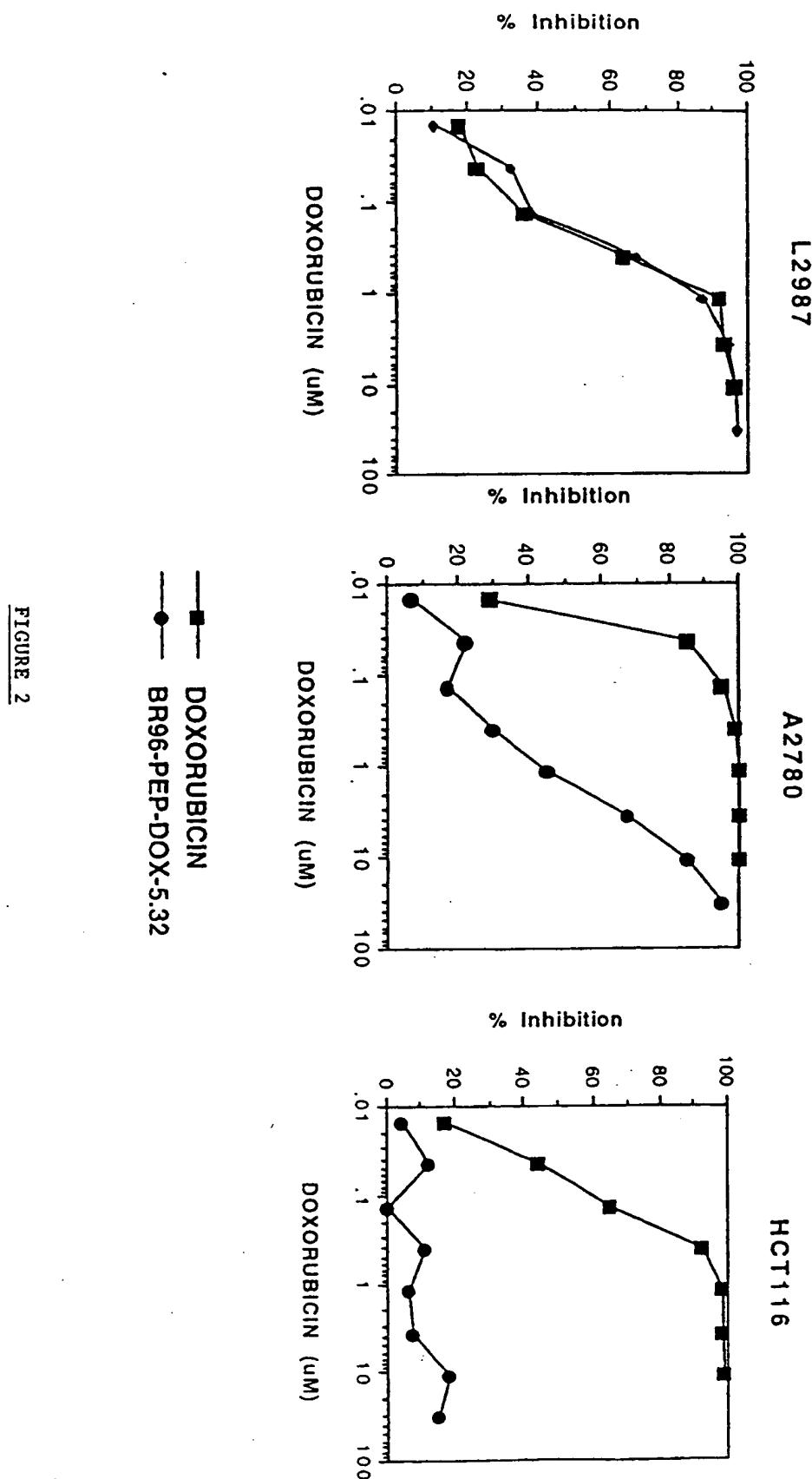


FIGURE 1

FIGURE 2



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(12)

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15.11.95 Bulletin 95/46

(54) Lysosomal enzyme-cleavable antitumor drug conjugates.

(57) The present invention relates to drug-ligand conjugates wherein the drug is linked to the ligand through a protein peptide linker and a connector, a process for the preparation of said conjugates, method of controlling the growth of undesirable cells, pharmaceutical compositions, and intermediates thereof.

EP 0 624 377 A3



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PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent Convention EP 94 10 7501
shall be considered, for the purposes of subsequent
proceedings, as the European search report

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.CLS)
X	WO-A-90 03188 (NEORX CORP.) 5 April 1990 * page 4, line 5 - line 32 * * page 6, line 3 - line 11 * * page 19, line 5 - line 26; claims 1,32,38 * ---	1-7,10, 11,13, 14,24-26	A61K47/48 C07K5/02 C07K5/06
P,A	EP-A-0 554 708 (SQUIBB BRISTOL MYERS CO.) 11 August 1993 * page 5; claims; figure 1 * ---	1-29	
A	WO-A-90 03401 (NEORX CORP.) 5 April 1990 * claims * ---	1-29	
X	US-A-5 013 547 (SWEET FREDERICK ET AL.) 7 May 1991 * claims * ---	1	
A	WO-A-88 00837 (BATELLE MEMORIAL INSTITUTE) 11 February 1988 * page 18; claims; figure IX * ---	1-29 -/-	
			TECHNICAL FIELDS SEARCHED (Int.Cl.5)
			A61K C07K
INCOMPLETE SEARCH			
<p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims</p> <p>Claims searched completely :</p> <p>Claims searched incompletely :</p> <p>Claims not searched :</p> <p>Reason for the limitation of the search:</p> <p>see sheet C</p>			
Place of search THE HAGUE		Date of completion of the search 11 July 1995	Examiner Berte, M
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background : non-written disclosure P : intermediate document			
T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			



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PARTIAL EUROPEAN SEARCH REPORT

Application Number

EP 94 10 7501

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.CLS)
			TECHNICAL FIELDS SEARCHED (Int.CLS)
X	INT. J. CANCER, vol.43, 1989 pages 677 - 684 NAOJI UMEMOTO ET AL. 'Preparation and in vitro Cytotoxicity of a Methotrexate-Anti-mm46 Monoclonal Antibody Conjugate via an Oligopeptide Spacer' * figure 1 * ---	1-5, 9, 11, 24-26	
X	J.N.C.I., vol.73, no.3, September 1984 pages 721 - 729 YUTAKA TSUKADA 'An Anti-Alpha-Fetoprotein Antibody-Daunorubicin Conjugate With a Novel Poly-L-glutamic Acid Derivative as Intermediate Drug Carrier' * figure 2 * -----	1	



EP 94 107 501 -C-

INCOMPLETE SEARCH

In view of the definition of products by means of their biological, chemical and or pharmacological properties, the search has to be restricted for economic reasons.

The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims or examples.
(See guidelines, Part B, Chapter III, paragraph 3.6)

Partially searched claims : 1-22, 24-29